

Iraq puts cat among pigeons

The arrest ten days ago of four would-be smugglers of bomb-making equipment, allegedly to Iraq, may mean more or less than is supposed, or is put about by interested parties. Yet nuclear proliferation deserves more worry than it gets.

THE cheerfulness of the past year, during which all the talk has been of impending agreements on arms control between the major powers, should not be banished by the drama at London airport at the end of last month, when four people were arrested on charges of seeking to transfer to an Iraqi Airlines flight a cache of 41 electronic capacitors of the kind used in the United States as components of imploding plutonium bombs. But the drama is a salutary reminder that while the major powers busy themselves with arms control in the framework of their mutual security, lesser powers are forever seeking to be counted among the majors. And there are ominous signs that some of these ambitions will succeed.

Not that the airport drama implies that Iraq is about to become a nuclear power. There are several reasons for that view. First, although Iraq has taken a serious interest in nuclear technology for the past 15 years, there is nothing to suggest that it is within a decade of being able to make even a few nuclear weapons out of its own resources. But then, of course, it will be different. But second, as the case of Israel has shown, it is no longer necessary to carry out a test explosion to be treated as a nuclear power. International speculation about the function of nuclear facilities hidden in the Negev may serve as well. So why should not one of Israel's neighbours seek the same effect by mounting a determined effort to secure illicit supplies of nuclear materials from elsewhere? The failure of the operation may, in that sense, imply that it succeeds. To the extent that international opinion will now be less certain of what Iraq is up to, and of what it has accomplished on the road to military nuclear power, there will be a tendency to regard it with greater awe. Spending a few tens of thousands of dollars on capacitors will, in the circumstances, prove to have been a bargain.

It is also relevant that the operation leading to the arrests in London, a cooperative effort between the Federal Bureau of Investigation and the Central Intelligence Agency in the United States and the Customs and Excise authorities in Britain, was a 'sting'. The smugglers, seeking illicit components in the United States, found themselves negotiating for their purchase with federal officials and their accomplices, who are for the time being the sole source of the speculation that the government of Iraq was behind the attempt to buy equipment in the United States. But those who conduct

stings are not the most disinterested witnesses. It is true that there is circumstantial evidence of Iraq's ambitions in nuclear and other warlike technology — the acquisition of ballistic missiles and the reasonably well substantiated use of chemical weapons against Iran in the recent Gulf War. But proof that Iraq, a signatory of the Nuclear Non-Proliferation Treaty (NPT), is about to make nuclear weapons is conspicuously lacking. If last week's drama at London Airport was not an elaborate bluff or double-bluff, the ultimate purchaser of the capacitors could just as well have been any one of half a dozen other countries.

That is the true cause for alarm. For at least the past three years, the company of putative second-string nuclear powers has been growing. Israel, whatever the truth about its nuclear capacity, has long since been accorded the respect due to nuclear powers — at least by its neighbours. Who can be surprised that they are now seeking to follow suit? Saudi Arabia, traditionally paymaster to the Middle East, is acquiring medium-range missiles from China. Kuwait, whose strong military links with Pakistan have endured many ups and downs, enjoys a little of the glory reflected from the spate of rumours of Pakistan's ambitions in the nuclear field (mercifully toned down by Ms Benazir Bhutto's presidency). Further afield, there are rumblings of putative nuclear power in India, South Africa and North Korea. If it were not for their burden of international debt, the two non-signatories of the NPT in South America — Argentina and Brazil — would be obvious other candidates.

All this has happened while attention seems to have been directed elsewhere. Have the major powers been indifferent to the dangers, or over-trusting in the arrangements they have created for restricting the transfer of advanced military technology? That is a complicated question. So far as it goes, the NPT seems now to be an effective means of preventing the accumulation of fissile material by its signatories. But there are two obvious weaknesses. There is no way in which the treaty's inspectors can prevent understanding accumulating in people's heads or laboratories, while the treaty itself will not outlast the time during which its non-nuclear signatories consider the benefits outweigh the disadvantages. The big prize has always seemed the nuclear powers' promise of progress towards the control of strategic weapons, which the Soviet Union and the United States seem ready to



deliver in the summer. But a serious defection from the treaty, which that of Iraq would be, could nullify even an agreement on strategic arms.

Other arrangements for restricting the spread of advanced military technology, the informal understandings between the major powers on nuclear and rocket technology and the equipment needed for making chemical weapons, are inevitably more leaky, as the past few years have shown. They cannot be a permanent safeguard, but only an impediment. Even so, there seems no ready explanation for the way in which small defence contractors in West Germany and Britain appear to be willing to turn a dishonest penny by selling forbidden technology, and at the same time appear to be privy to enough secret information to realise their ambitions.

What, in these circumstances, can be done? The most urgent need is that China and France, nuclear powers which have chosen not to sign the NPT, should be tempted into the club. It would be splendid if that could be done by September, when the fourth quinquennial review conference of the treaty is due. But that step is neither necessary nor sufficient. French objections, unfashionably gaullist as they are, no longer carry much weight even in France. China will evidently be a harder nut to crack, especially now that events in Beijing a year ago have led to an embargo on the supply of advanced 'conventional' military technology — aircraft and the like. To create the climate in which to win China round to membership, the other nuclear powers may have to follow a self-denying ordinance on the sale of arms of all kinds. That would be no bad thing. □

Abortion from a hat

The British government is making a quick stab at abortion legislation in what may be the worst possible way.

TOWARDS the end of last year, the president of the Royal Society, Sir George Porter, wrote to the London *Times* to plead that the issue of artificial abortion should not be confused with the issues raised by the Embryo Bill, then beginning its journey through the British Parliament. The hope was that calm consideration of the legislation to regulate research with human embryos would not be prevented by the emotionally charged arguments habitually assembled for and against abortion. There have always been reasons for believing that the hope would be disappointed, while the Embryo Bill, which proposes a 14-day time limit for experiments with embryos, will almost certainly be used as an argument against abortion when the bill becomes law. But now the British government has startled the House of Commons by announcing that it intends to tag onto the Embryo Bill an amendment that would fix the legal time-limit for abortion at 24 weeks rather than the present 28 weeks.

The decision is both mystifying and deplorable. The Embryo Bill is the government's belated attempt to give effect to the Warnock proposals on embryo research, now six years old. There is an urgent need of it. Although opinions differ on some aspects of the bill, the sanctity of the 14-day limit for example, it is the best that is likely to be had from this parliament, which has just over two years at most to run. So why should the government complicate and perhaps even jeopardize the passage of its own legislation by converting that into a trojan horse for the carriage of an amendment of abortion law? Opportunism seems to be the simplest answer.

As the technology of medicine has advanced, the case for reducing the time-limit for abortions from the present 28 weeks has been strengthened. The diagnosis of pregnancy has been simplified and made more accurate, while the survival of early fetuses is more easily assured. Two years ago, the government was attacked for failing to use its powers to assist legislation put forward by Mr David Alton, MP. More recently, it has failed to lift a finger to help through the House of Commons legislation successfully introduced in the House of Lords by Lord Houghton. Until now. By all accounts, the government's intention is to incorporate the essence of Lord Houghton's Bill into its amendment, which is to be debated later in the month.

The case for some such amendment is not negligible, but the course now proposed is unsatisfactory. Its most serious defect is that it allows no time for public discussion. The reason why abortion is a contentious issue is that the available spectrum of opinion is spanned by warring lobbies whose arguments, while cancelling out each other, make all compromises unacceptable. At the extremes are the views that all abortions are acts of murder and that women, uniquely susceptible to pregnancy, have a right individually to choose what happens to their own bodies. But even within the framework of the existing legislation on abortion, much has been done by intelligent medical practice to avoid late terminations. There would be a case for new legislation on the issue only if there were reason to believe that present legislation should be scrapped or if some objective and thorough study of the working of the present system had suggested that a more intelligent compromise than the present could be reached. Neither of those conditions has been met.

So what will be the outcome? The government may mollify some of those who have criticized its inactivity on the Alton bill, but ironically will probably offend a greater number who hold the Houghton measure to be milk and water. (By counting from the supposed time of fertilization, taken as two weeks after the last menstrual period, the Houghton bill reduces the present time limit by only two weeks.) But one thing is certain — the Embryo Bill will be further delayed by the need for another long discussion in the House of Lords. But worse, on yet another contentious issue, the prospect that a more intelligent compromise might be reached after discussion has been buried under a government decree. □

US takes the offensive on animal research

- Scientists will be paid to oppose activists
- Controversial memo re-emerges

Washington

US HEALTH officials, facing an increasingly effective and sophisticated animal-rights movement, have begun an unprecedented, multi-million-dollar effort to promote the use of animals in research.

A new draft plan prepared by officials within the US Public Health Service (PHS) outlines a wide-ranging effort to fund 'outreach' and educational efforts to counter the anti-animal-research campaigns of groups such as People for the Ethical Treatment of Animals (PETA).

"The use of animals in biomedical research is indisputably part of the fundamental philosophy of the [PHS] and we will articulate that and explain it to the public as we do everything else we do", says Frederick Goodwin, director of the Alcohol, Drug Abuse and Mental Health Administration (ADAMHA). "To sit back and ignore the [animal rights] movement, hoping that it will go away, would be the height of irresponsibility." The National Institutes of Health (NIH) and ADAMHA have already created a new joint Office of Animal Affairs that will coordinate some of the first efforts. Located on the NIH campus, the office will take its initial funding from the research-training and public-affairs budgets of the individual institutes within NIH and ADAMHA.

Goodwin says the two agencies will ask for funding "in the millions of dollars" in their 1992 budget for the programme. Matching funding from private organizations such as the Hughes Foundation is also being discussed, as is the involvement of groups such as American Medical Association and the National Association for Biomedical Research.

One of the new office's first actions will be to solicit grant applications from working researchers willing to take a sabbatical to speak to high-school and pre-high-school students about the importance of animal research. Goodwin says that he expects that 10-15 full or part-time awards will be made in the first year. Funding would be available to continue the scientists' research while they are gone. "The intent is to allow them to do this without having to shut down their labs", he says.

Although the efforts will be only one part of a major new PHS educational initiative to increase scientific literacy in pre-college students, the increasing number of attacks and break-ins by animal-rights extremists has created "a

need to take an advocacy position" and highlight animal research, according to the draft PHS report.

"The scientific community had allowed itself to slip into a reactive posture, in effect answering charges from the activists", says Goodwin. "I know of scientists who have taken time to enter their local high schools and encountered stunning ignorance and stunning prejudice because PETA had been there first. This is not an issue of what's right and where the truth is; it's an issue of how to convey the truth, and we're way behind." Many of the components of the programme have their origins in a controversial 1987 memorandum from Goodwin (then director of intramural research at the National Institute of Mental Health) to his superiors. Stressing that the "bunker strategy" was no longer an effective defence against animal-rights activism, Goodwin suggested "special fellowships in research advocacy". The Department of Education "should be contacted concerning infiltration of high schools by the animal rights people. PHS should sponsor counter-educational efforts", he wrote.

Although animal-activist groups widely quoted the memorandum as evidence of conspiratorial behaviour within the PHS, Goodwin still stands by the thrust of the document. "Fundamentally the department has come around to what I was saying in 1987", he says. "When I did that memo I was in no position to [do anything but] stimulate ideas in the people who were in a position to do something. Now I'm in a position to do something." He suggests that the PHS-funded research advocates mince no words in their characterization of the animal-rights community. "Unless we educate the public about what the agenda of the other side is, they will tend to take [animal-rights statements] at face value, particularly if the person saying it has a MD or a PhD", he says.

ADAMHA has distributed a set of Goodwin-prepared slides and 'talking points' to 700 people. The package "identifies the fundamental philosophical position [of the animal-rights movement] and takes it on", he says.

The package discusses the origins of the movement, including the "gradual take-over and radicalization of animal welfare organizations". It lists some common animal-rights arguments ("Modern alternatives [such as computers] can replace animals in research"). And it outlines some suggested responses ("Would you

JAPANESE SCIENCE

New science institute gets director

Kyoto & Tokyo

WITH effect from 1 April, Michio Okamoto, one of Japan's leading academics and a key advisor to the government, has been appointed director of the new International Institute of Advanced Studies (IIAS).

IIAS will form the 'brain centre' of the new Kansai academic city being established between Kyoto, Nara and Osaka. The science city is expected eventually to have a population of 400,000 people and will cost \$20,000-\$25,000 million to build (see *Nature* 338, 285; 1989).

IIAS was established as a foundation in 1984 with Azuma Okada, a former president of Kyoto University, as chairman of the institute's board of trustees. The institute, construction of which will begin in a few months with completion expected in spring of 1992, will be modelled on the Centre for Advanced Studies at Princeton in the United States, according to Saburo Fukui, chairman of the planning committee of IIAS. It will have a constantly changing population of about 40 senior academics from all over the world drawn from both the arts and the sciences supported by a small permanent staff at the institute. The researchers will be housed in a 'scholars' village' surrounded by landscaped gardens where they can contemplate the future of science and the world.

Okada, realizing that the Japanese government is heavily in debt, has turned to Japan's private sector and a few rich stockholders of major companies to fund the institute. Fifty million dollars has been raised so far and the government has provided support by making donations to the institute tax-deductible.

Okamoto was formerly chairman of the academic committee which mapped out plans for the international Human Frontier Science Program and he has also served on various government committees including the Council of Science and Technology, Japan's science policy-making body chaired by the prime minister.

Simon Wallis & David Swinbanks

take a drug that had only been tested on a computer or in a tissue culture?").

Although there are no plans to make the package a formal part of the programme, most researchers who apply are likely already to have a copy, Goodwin says. Michele Applegate, director of the ADAMHA office of external affairs, says: "Our goal is to be blunt, forthright, and to counter specific arguments made by the opposing groups". Expected targets of the education initiative will be the public, educators, legislators, the biomedical community and professional societies, says Louis Sibal, director of the new animal research office.

G. Christopher Anderson

Juno's coffers bare

London

THE Juno project, an Anglo-Soviet venture to send a British astronaut and a series of low-cost microgravity experiments to the Mir space station in April 1991 (see *Nature* **343**, 107; 11 January 1990), is in disarray following the withdrawal of its original financial backers, the Moscow Narodny Bank.

Unlike the earlier Soviet collaboration with the French, and the plan to put the first Austrian in space in late 1991, Juno has received no government support, and has looked to commercial sponsors to cover the £16 million British contribution. The London-based Moscow Narodny Bank has provided 'seed finance' for the mission for the past nine months, according to its deputy chairman, Sergey Konychev. But Konychev says the bank will provide no more money except to satisfy any legally binding commitments, including paying the trainee astronauts' salaries.

Konychev estimates that only about £2

Hipparcos in the dark

London

THE Hipparcos astronomy satellite has emerged successfully from a series of long solar eclipses, drawing from on-board batteries when its solar panels were shadowed by the Earth. The eclipses became inevitable once the satellite became trapped in an elliptical orbit, rather than the planned geostationary one, after its launch last year. During the longest eclipses, some ancillary equipment was shut down, to enable data collection to continue. Even so, an eclipse just five minutes longer than the maximum, on 16 March, would have interrupted measurements.

Although Hipparcos has survived this physical challenge, a decision on the source of future funds for the project has been deferred until October. The European Space Agency (ESA) council, has decided to set up a high-level working group to consider the 'Pinkau' review of ESA's Horizon 2000 science budget (see *Nature* **344**, 280; 22 March 1990). The Pinkau report recommends further budget increases, and proposes reforms for the Horizon 2000 programme, including freer competition for contracts awarded by ESA to equipment manufacturers. These are at present awarded to subscriber states in strict proportion to their ESA subscriptions.

A decision on whether to ask ESA subscribers for more money must wait until the working group reports back. From July, when Hipparcos's present budget runs out, until October, money will have to be found from within the existing Horizon 2000 budget.

Peter Aldhous

million had been raised when the bank withdrew last week, and negotiations in progress looked unlikely to raise this figure beyond £6 million. He is pessimistic about the chances of finding new sponsors in the absence of any agreed television coverage — independent television companies have pulled out of a deal rumoured to be worth £3 million, following Moscow Narodny's withdrawal from the project. The other manned mission proceeding without government funding is a Japanese-Soviet collaboration funded by a Japanese television company, Tokyo Broadcasting System.

Sir Geoffrey Pattie, chairman of Antequera, the company organizing fundraising for Juno, confirmed last week that the lack of interest from British companies meant that Juno cannot continue in its current form. Jack Leeming, from Antequera, last week met with representatives of Glavkosmos, the Soviet space agency, at a space commerce conference in Montreux, Switzerland, to negotiate a modified mission.

Professor Heinz Wolff, Juno's science director, says that "every avenue will be explored" to continue the mission. He says the Soviets have an interest in the planned microgravity research, and also appreciate the goodwill value of launching a British astronaut. But this seems unlikely to change to a willingness to fund the British end of the project.

Wolff says that, for the time being, the £2.3 million science programme will continue unchanged, and the British astronauts, Tim Sharman and Helen Mace, will continue their training in the Soviet Union. But he expects a 'crunch time' within the next month. One possibility being considered is to abandon plans to put a Briton in space and to fly some of the microgravity experiments on other missions.

Douglas Hogg, Trade and Industry Minister, has confirmed that the British government will not rescue the Juno mission.

Juno's failure to interest British companies, despite the media circus accompanying the astronaut selection, augurs badly for another British space project seeking sponsorship. Nina, the British design aiming to enter the 'space sail' race to Mars in 1992 (see *Nature* **344**, 185; 15 March 1990), needs £7 million. Cambridge Consultants, the craft's designers, have been told by race organizers that they have "the most advanced technical design", but seem likely to lose out to an Italian design as the favoured European entry. The Italian team has already negotiated sponsorship and have the republic's president as the honorary head of the project.

Peter Aldhous

War is unhealthy, US finds

Washington

AFTER five years of study, the US Centres for Disease Control (CDC) last week concluded that there is no evidence that the chemical defoliant Agent Orange was responsible for causing cancer in US Vietnam War veterans. But the agency did find that in the case of one rare disease — non-Hodgkin's lymphoma — Vietnam veterans showed a 50 per cent higher risk than other men in their age group. It is the first time the US government has acknowledged a link between cancer and service in Vietnam.

Paradoxically, the study showed that the two groups that exhibited the highest incidence of lymphoma were ground troops stationed in areas of lowest Agent Orange use, and sailors in Navy ships based off the coast of Vietnam. Navy veterans who served elsewhere showed no such elevated cancer rate. "The pattern of risk among subgroups of Vietnam veterans seemed to be the opposite of the pattern of use of Agent Orange", CDC reported. Initial speculation was that an anti-malarial drug could be to blame for the increased lymphoma rates, but that has since been discounted. No other explanation has been offered.

Although the study had originally been intended to settle the question of whether Agent Orange was responsible for cancer cases in Vietnam veterans (which would make them eligible for service-related disability payments), early results showed that Agent Orange exposure, as measured by dioxin levels in fat, did not correlate with service records. Few of the veterans who showed raised dioxin levels had handled Agent Orange or travelled through defoliated areas shortly after the areas had been sprayed.

Because the Agent Orange study had been approved contingent on some reliable method being found to determine exposure, that portion of the study was dropped in 1986. Since then, it has been described as a 'Vietnam experience' study, with no particular carcinogen or disease as its target. The CDC researchers compared more than 2,000 cancer cases in US men who were of fighting age during the Vietnam War with 1,776 men in the same age group who did not have cancer. Details of military service were self-reported. Few of the reported data were verified with service records. CDC checked only 70 per cent of the self-reported veterans to see if they had actually served in Vietnam.

Following the release of the CDC study, the US Veterans Affairs agency announced that it would add non-Hodgkin's lymphoma to its list of 'service-connected' diseases. Between 1,600 and 1,800 Vietnam veterans will receive disability payments.

G. Christopher Anderson

Plunging yen hits grants

Tokyo & London

THE first international teams of scientists to win grants under Japan's Human Frontiers Science Programme, whose names will be announced this week, may be disappointed to find that they have not been awarded as much money as expected, because of the recent dramatic fall in the exchange rate of the yen.

Frontiers was established as a foundation in Strasbourg last year with a budget of ¥2,400 million for fiscal year 1989 to support international research on the brain and molecular biology. In 1988, when the Science and Technology Agency (STA) and the Ministry of International Trade and Industry (MITI) applied for the budget, this was worth nearly \$20 million at the then exchange rate of ¥123 to the dollar. But since then, the yen has gradually fallen in value, nose-diving in the past few weeks to ¥159 to the dollar. This has sliced \$4.3 million (22 per cent) off the original value of the budget.

When grant applications were invited last year, applicants were told they could request up to \$500,000 a year for three years (a figure recommended by the Frontiers feasibility study committee in 1988). But last week, Toichi Sakata, director of STA's Frontier office, revealed to *Nature* that the maximum grants have had to be limited to \$400,000 a year, partly because of the fall in the value of the yen. The size of the grants also had to be reduced because more grants have been awarded than at first intended.

The original plan was to award about 20 grants in the first year, but the programme was flooded with over 200 applications (see *Nature* 343, 684; 22 February 1990). And at a meeting in Strasbourg on 16 and 17 March, the chairmen of the two grant review committees (for the brain and molecular biology) put forward 60 applications which were rated as being of top quality and well worthy of award.

A heated debate then ensued about how many grants to award and the council of scientists finally chose 29 grants and allotted them budgets of between about \$200,000 and \$400,000 per year. Several will receive only about 50 per cent of what they originally requested. And, according to one, some projects may now be "non-viable". But others are grateful for any new source of funds.

The 29 grants go to teams made up of a total of 159 scientists. The biggest contingent (45) is from Japan, followed by the United States (36), France (23) and the United Kingdom (20). Eighty long-term postdoctoral fellowships have also been awarded, with the largest number (18) going to Japanese scientists, followed by the British (17).

One Japanese scientist close to the pro-

gramme criticizes for its lack of transparency the screening process used to select successful applicants. Unsuccessful candidates may believe they were overlooked for reasons other than scientific criteria, if these are not made clear. Last year a small 'pilot' Frontiers grant programme run by MITI's New Energy and Industrial Technology Development Organization was criticized by some unsuccessful applicants because they were given no explanation for rejection, no rating of the quality of their application and no access to referees' comments. This year's full-scale programme may receive similar complaints. Raymond Bahor, from the programme's Strasbourg office, says that the 210 teams that made unsuc-

cessful grant applications will not be given a rating of their applications or reasons for rejection. He says the office does not have sufficient staff to do the job.

Sakata says that there are bound to be teething problems in the first year of full-scale operation of the programme. Complaints and criticisms are inevitable, and in fact welcome. He admits that programme organizers were caught off guard by the plummeting value of the yen, because they were so busy screening applications. Although some funds have been transferred to a bank account in Strasbourg and converted to French currency, most of the fiscal 1989 budget is still in a bank in Japan in yen. In future, he says, they will consult financial experts on how best to play the international money market.

David Swinbanks & Peter Aldhous

MOUNT GRAHAM TELESCOPE

Judge halts construction

Washington

WHAT has become one of the most contentious scientific projects in recent memory took a surprise turn for the worse last week as a federal judge halted construction of the proposed Mount Graham astronomical observatory.

Environmental activists have protested that the \$200-million telescope complex would threaten about 150 endangered red squirrels that are unique to the proposed site atop the 10,700-foot Mount Graham near Tucson, Arizona.

US district judge Alfredo Marquez said he called a four-month stay on construction of the project to allow Congress to reconsider its 1988 decision to exempt the project from the US Endangered Species Act. Championed by the Arizona delegation, the congressional bill gave the University of Arizona a 'special-use authorization' to go ahead with the observatory before all environmental-impact studies were completed.

But since the passing of the legislation, several events have raised new questions about the observatory. Several environmental groups have sued the government. In a court deposition for the case, two Fish and Wildlife Service biologists testified earlier this year that pressure was put on them by their superiors to conclude that the observatory would not threaten the squirrels. Critics have charged that the University of Arizona had 'railroaded' the congressional legislation with a massive lobbying effort. And more than a third of the members of the university's ecology department recently signed a letter criticizing the institution for "scorning the few laws that do exist to protect our environment".

Although the Arizona Fish and Wildlife Service is considering preparing another

'biological opinion' on the impact of the observatory, it is making no commitments. In May, when the snow has left the mountain, the service will begin a census of the remaining squirrel population. "We don't know yet if the biological situation has changed", says Tom Smylie, assistant regional director for the agency.

University officials point out that 5.5 of the 8.6 acres of land the observatory would occupy has already been used to build a road leading to the site. "There has been no biological impact that we can detect at this point", says Michael Cusanovich, vice president for research. The university has appealed against last week's court decision, and requested an expedited ruling.

A spokesman for Representative Jim Kolbe (Republican, Arizona) says the congressman would consider removing the university's exemption from the Endangered Species Act if another biological opinion came to a different conclusion from the first. And in a radio interview last week, Senator John McCain (Republican, Arizona) suggested that it might be time to have oversight hearings to reexamine the 1988 bill. Opponents of the project say that there are at least four alternative sites in the world where the telescope would have no adverse environmental impact.

If the university appeal is denied, the court-ordered stay could mean a substantial delay for the observatory, says project leader Peter Strittmatter. Because the construction season on the mountain is shortened by snow, a four-month postponement could set the project back an entire season, he says. A congressional reversal of the 1988 Endangered Species exemption could set it back even further.

G. Christopher Anderson

Plunging stock market halts break-up

Tokyo

THE plunging Tokyo stock market has put an end to plans to break-up Japan's domestic telecommunications giant, Nippon Telephone and Telegraph (NTT), at least until 1996.

Last month, an advisory committee to the Ministry of Post and Telecommunications recommended that to introduce fairer competition in the domestic telecommunications market NTT should be broken up into three separate companies, one to handle local services, one for long-distance telecommunications and a third for mobile telecommunications. The ministry expressed its determination to carry out the plan despite strong opposition from the Ministry of Finance, the major stockholder in NTT, industrialists, NTT union members and politicians in both the ruling and opposition parties (see *Nature* 344, 370; 29 March 1990).

The Ministry of Finance was concerned that future sales of NTT shares to the public, a major source of government revenue, would be adversely affected by the break-up. And no sooner was the break-up plan announced than the Tokyo stock market went into its second nosedive of this year, dragging down NTT stock, the highest priced shares on the market, to their lowest value ever.

When the government sold the first batch of NTT shares in 1986 they were priced at ¥1.197 million each (about \$7,500 at the current exchange rate). A year later, the price had soared to ¥3.18 million and the government raked in thousands of millions of dollars from the public when a second batch of shares was sold. The funds were invested in public works.

But since then the price has been falling steadily and with last month's stock market crash the stock reached an all-time low of ¥1.06 million. Some analysts say that by releasing the shares in dribs and drabs, the finance ministry, which still holds two thirds of NTT's stock, inflated the value of the shares and swindled the Japanese public, many of whom have lost tens of thousands of dollars through buying NTT shares at their peak price.

Whatever the case, the recent crash in the price of NTT shares gave the ministry of finance and other NTT supporters the chance to strangle calls for break up. The small mobile telecommunications section of NTT will probably be separated in the next year or so. But all talk of separating the local and long-distance operations of the company have been shelved until at least the end of fiscal year 1995 (March 1996) and the Japanese public can look forward to continuing to pay some of the highest domestic telecommunication charges in the world.

David Swinbanks

Science and social renewal

Moscow

THE annual general conference of the Soviet Academy of Sciences broke new ground this year by the attendance of representatives of academic institutes and of the union of workers of public education as well as academicians and corresponding members. Academy vice-presidents V. Kudryavtsev, K. Frolov, O. Nefyodov and V. Koptug, the main speakers, tackled the novel theme (for a general conference) of the role of science in social renewal. *Perestroika*, the speakers said, needs a solid scientific foundation. But science must win back the prestige it has lost over the long years of dogma-ridden stagnation and during the five years of *perestroika*, which have seen few meaningful changes either in the socio-economic sphere or in science and technology.

Social sciences were much criticized. Developments in the West have been overlooked for ideological reasons, and interest in world science has not been encouraged. Dogmas have obliged scientists to provide scientific backing for the policies of the political leadership. *Perestroika* may have persuaded scientists to re-evaluate their methods and to break many stereotypes, but this process has just begun. A new network of sociological research centres is in place, but plans to organize an institute of the socialist market and a research centre for socio-economic, scientific and technological development are still to be implemented.

Many academy members are involved directly with *perestroika*. The institutes of economic, philosophical, legal and sociological studies are drafting documents for the USSR Supreme Soviet, and there are 59 scientists among the People's Deputies of the USSR, from which the members of the Soviet are drawn. Time will show whether Soviet scientists are able to make radical use of their opportunities to shape Soviet society. But this is a scarce commodity and scientists must quickly regain the nation's trust.

The meeting acknowledged that, in science and technology, the Soviet Union lags behind the Western industrialized states. The unsatisfactory position of science in society, a separation from industry and a dire equipment shortage were blamed. The Soviet economy still resists technical innovation. Last year alone, fundamental research centres proposed 3,000 new ideas and types of equipment, but only a fifth are being used. New problems have arisen. In 1989, the academy's president, G. Marchuk, said, the academy felt its first financial squeeze. Although academic science funding increased by 50 per cent last year, the economic crisis is damaging research programmes. Academic institutes have

suffered in the uncertain climate of *perestroika*, as ministries have switched to self-financing schemes, cutting back research programmes that do not promise fast returns. Research in plasma physics, thermonuclear fusion, earthquake forecasting and space exploration are threatened.

Support for fundamental research from defence-related industrial branches, formally an important source of funds, has been cut. The Institute of the Optical Properties of the Atmosphere of the Academy's Siberian branch had been fulfilling orders for the defence branches for 20 years, said its director Academician V. Zuyev. Permanent clients, he said, were well aware that financing applied science programmes exclusively was not useful in the long term. But this changed suddenly, when contracts to last until 1992 were annulled without the right of appeal.

Speakers also argued that there is little genuine competition for funding in the academy. Project funds are divided equally among many research centres, rather than being awarded on merit.

A 'brain drain' and the falling prestige of researchers reflect the vulnerability of Soviet science, speakers suggested. By world standards, wages are low and poor equipment affects morale. In 1989, 246 associates of academic institutes left for a term abroad of up to five years. This process would not hamper Soviet science if it were two-way, but it is not. The meeting asked the academy's presidium to study the problem and regulate foreign trips. This may mean a return to restrictions. A more democratic approach would be to create favourable conditions to attract foreign scientists and young Soviet researchers, many of whom escape via a second brain drain: to the new cooperative ventures, where wages are higher.

The meeting highlighted the sores of Soviet science and its decisions were adopted unanimously. But there are worries that, as in the past, resolutions will not be implemented. The academy is calling for greater state support, and a legal framework to give scientists a protected position in Soviet society. These claims may be justified, but it is unclear if the state has the necessary resources. Although the adoption of a Law on Science is expected, the Supreme Soviet has a busy schedule. The meeting also resolved to return academy membership to scientists expelled for political reasons, mostly under Stalin's regime, and to commemorate scientist's who were victims of repression. A memorial is to be built and a collection of biographies is planned for 1991. The academy also suggested naming streets in Moscow and other cities after scientists destroyed by Stalin. **Yuri Kanin**

Novosti Press Agency

Agreement questioned

Washington

THE allegation that the US government has concealed the controversy about the discovery of the human immunodeficiency virus (HIV) is the latest to be levelled by John Crewdson of the *Chicago Tribune* in his continuing commentary on the AIDS controversy. The latest allegations have sparked calls to renegotiate a 1987 treaty in which the United States and France agreed to share credit for the breakthrough. But it is still not clear whether such a renegotiation is legally justified, or whether the latest details were already known to the signatories of the treaty.

Crewdson, who last November wrote a 50,000-word article alleging that Robert Gallo of the US National Cancer Institute (NCI) obtained the AIDS virus from Luc Montagnier of the French Institut Pasteur either by "accident or a theft", wrote a further article on 25 March alleging a US cover-up of the matter.

Quoting a 1985 memorandum from Peter Fischinger, then NCI associate director, to a top official in the department of Health and Human Services (HHS), Crewdson points to "misstatements and omissions" that he says could have deceived the French into agreeing to share the discovery. The document is one of the few that the National Institutes of Health (NIH), under which NCI falls, has refused to release to Freedom of Information Act requests. Although Crewdson does not reveal how he got the memorandum, informed sources say it was leaked by a former government official.

Comparing the Fischinger memo with the Gallo laboratory records and other documents, Crewdson concludes that the memorandum selectively chooses some facts and ignores others to make a stronger argument for Gallo's discovery of the AIDS virus. Because the disputed memorandum was an important part of the documentation that led the US Justice Department to conclude that Gallo had independently discovered the virus, Crewdson suggests that the US case did not accurately reflect the facts known at the time. The French, who had not seen the memorandum nor the documentation leading up to it, may have thought that the case for an independent Gallo discovery was stronger than it actually was, he says.

Lawyers for the Institut Pasteur say that the revelations could be grounds for renegotiating the treaty. Among the factors that will be considered is how much information had been withheld from the French in 1987. "Was Pasteur significantly misled at the time?" asks Ira Millstein of Weil, Gotshal & Manges. He says the Pasteur lawyers are discussing the issue with NIH.

But one member of the team that

negotiated the 1987 treaty believes that there is no basis for a reevaluation. "It was agreed that the [NCI] test kit was based on information gleaned from LAV [the Institut Pasteur version of the virus]", says Robert Charrow, a lawyer who was then in the HHS general counsel's office. Because the signatories had already acknowledged that Gallo's work had incorporated Montagnier's virus, "By definition [the treaty] could not be renegotiated." He believes the Fischinger memorandum (which he has seen) adds nothing new.

Michael Astrue, HHS general counsel, says he is familiar with the allegations, but he would not comment on the possibility of renegotiating the treaty.

The congressional oversight and investigations subcommittee under Representative John Dingell (Democrat, Michigan), which has taken an active interest in the matter, has so far made no moves in reaction to the latest disclosures. With an NIH inquiry in progress (see *Nature* 343, 680, 22 February 1990), Dingell is expected to wait for a report from the investigators before taking new action.

In response to a January letter from Dingell, NIH director William Raub outlined 14 specific allegations the agency's Office of Scientific Integrity was investigating. Raub said that attention would be focused on "questions about how many isolates [came] from AIDS patients and when this occurred" in Gallo's laboratory, and "questions about the H-9 cell line used" in the laboratory.

Although Crewdson's account has earned surprising little press attention in the United States, the French press has been riveted. Montagnier has been quoted in *Le Monde* as appealing to Gallo to "at least accept the evidence" and admit the true source of the AIDS virus. Such statements may threaten the treaty as much as the Crewdson allegations.

Attached to the 1987 document is a 'scientific history' of the discovery of the AIDS virus (*Nature* 326, 435; 1987). And in the text of the treaty is a clause stating that Robert Gallo and Luc Montagnier agree not to "make nor publish any statement which would or could be construed as contradicting or compromising the integrity of the said scientific history". Pasteur lawyer Millstein says he "did not advise Montagnier to make those statements". Nevertheless, the contrary reactions from opposite sides of the Atlantic suggests that the debate may well intensify. As the NIH, with the help of a new 11-person committee selected by the National Academy of Science, continues its secret inquiry, one observer wryly notes "in Paris, they're rioting in the streets".

G. Christopher Anderson

Leading biotechnology companies quit the IBA

Washington

LAST week's resignation of Genetics Institute and Cetus Corporation from the Industrial Biotechnology Association (IBA), following shortly after the withdrawal of another member, the Upjohn Company, is seen as a major loss to the association. Both companies to leave last week were founding members of the IBA in 1981 and are prominent in the industry. In a letter to Richard Godown, president of the IBA, Robert Fildes, president and chief executive officer of Cetus, stated that "the IBA has too frequently become the mouthpiece for a few companies whose positions do not represent those of all the members". The final straw seems to have been a vote taken by board members on 21 February to oppose changes in the 1983 Orphan Drug Act, as well as to support a bill now before a House of Representatives subcommittee, which proposes to strengthen US patent protection for genetically engineered products against unfair foreign competition.

The vote was unanimous, although neither Gabriel Schmergel, chief executive officer of Genetics Institute, or Fildes of Cetus, were present because of prior commitments. Both companies support changes to the act and are opposed to the proposed patent legislation. In a joint statement, they said that the act stands to "perpetuate or create market monopolies for highly profitable products such as recombinant human growth hormone and erythropoietin", two products with orphan drug status. Katharine Russell of Cetus says "to see the trade association take an anti-competitive position" on these matters is "lamentable".

The Orphan Drug Act was designed to provide tax incentives and a seven-year marketing monopoly in the United States to companies developing drugs for rare diseases affecting fewer than 200,000 patients.

Also at issue is the 'Boucher bill', introduced last month by Representative Rick Boucher (Democrat, Virginia). The bill would provide the International Trade Commission with the authority to exclude foreign products made using a host cell, DNA sequence, or vector patented in the United States, closing what the bill's sponsors believe to be an unfair legal loophole.

Genetics Institute would be affected by the legislation, as it would prevent the importation into the United States of its EPO product, which is produced in Japan by its licensee, Chugai Pharmaceuticals.

Godown was both "surprised and saddened" by the news and says that "this dispute arises out of a difference of opinion concerning what is best for the long-term interests of the development of the biotechnology industry".

Diane Gershon

Chemical companies rebel

Boston

THE Bush administration's plans to modernize the United States' chemical weapons stockpile have stalled, according to information that surfaced from the Pentagon last week, because the two major domestic manufacturers of a key chemical ingredient have declined on ethical grounds to produce it. As a result, the administration must either forfeit this year's funding for the chemical modernization programme, or face the potential embarrassment of forcing the companies to sell the chemicals to the military.

Ironically, the US administration is now finding itself thwarted by the same strengthened corporate controls on restricted chemicals that it has long advocated to halt chemical-weapons proliferation abroad. Citing their own tightened corporate policies prohibiting the sale of materials for use in chemical weapons, the Occidental Petroleum Corporation and the Mobay Corporation have both declined to produce the chemical thionyl chloride for the Defense Department.

US officials must now decide whether to allow the Commerce Department to invoke a law dating from the outset of the Korean war that would compel the US manufacturers to comply with the military's purchase order.

Thionyl chloride is an internationally restricted ingredient needed for the production of the nerve agent used in the Army's 155 millimeter binary artillery shells. Last spring, the US administration

moved successfully to block the sale of thionyl chloride by a West German company to Iran. Until several decades ago, the US Army itself produced the chemical at a now-closed facility. But a Pentagon official said that stocks of the substance were depleted earlier this year, requiring the purchase from a private vendor.

To receive a congressionally authorized \$47 million for a special weapons-modernization programme, the Army must establish that its Pine Bluffs, Arkansas, chemical-weapons production facility meets federal requirements and will open on schedule this year. To do this, Army officials say, the facility will need to procure 160,000 pounds of thionyl chloride by June.

A statement released by the Occidental Petroleum Corporation asserts that company policy prohibits it from selling or distributing chemicals "that contribute to the production of chemical weapons or illicit drugs". A similar sentiment is echoed by Gerd Wilcke, a representative for the Mobay Corporation. Wilcke says that the Army acknowledged that thionyl

chloride would be used in chemical weapons when it first asked Mobay last autumn for the material. "Such a use would violate our corporate policy", Wilcke said, "and that is why we refused." Mobay's chief executive officer, N. H. Prater, has stated that his company has "no intention of violating the law" but would not make the delivery of the material without considering its legal options.

The situation is complicated because Mobay is a US subsidiary of Bayer AG, West German chemical company. In fact, given tightened West German export regulations adopted after public outcry over chemicals sold to Libya (*Nature* 337, 678; 23 February 1989), it is conceivable that West German nationals working for the US subsidiary involved in a transaction could even be prosecuted under West German law.

Kyle Olson, a spokesperson for the US Chemical Manufacturers Association, says that the companies adopted policies on the matter as part of a voluntary international programme adopted last autumn by most of the world's major chemical companies to prevent the diversion of their products for use in chemical weapons (see *Nature* 341, 271; 1989).

Seth Shulman

HEALTH RISKS

Radiation research revamped

Washington

IN the last of his major changes to the troubled US Department of Energy (DOE), Energy Secretary James Watkins last week opened much of the department's health monitoring and radiation-research activities to private investigators and US health agencies.

Long-term research into the health effects of radiation on current DOE workers will be transferred to the Department of Health and Human Services (HHS), which runs the National Institutes of Health and the Centers for Disease Control. Health data from former and current workers will be compiled in a national database and will be available to independent researchers, Watkins said at a press conference.

The moves follow the recommendations of a final report from an independent advisory panel set up by Watkins last year. The report was also released last week. Kristine Gebbie, who headed the panel, said that widespread "concerns about credibility" had dominated the committee's thinking (see *Nature* 344, 92; 8 March 1990). Only by shifting the responsibility for some of the more sensitive health research out of DOE can the agency avoid the perception of conflict of interest among its workers, she said.

But "merely transferring the [DOE epidemiology] program in total would

overlook the facts that no employer can transfer its responsibility to protect worker and community health", the report warns. It proposes a compromise, in which 'analytic' epidemiology would be transferred to HHS, while 'descriptive' epidemiology remained within DOE.

Analytic epidemiology, as defined by the panel, is research designed to test causal hypotheses. Because this kind of analysis requires that scientists develop hypotheses without concern for the consequences should they turn out to be true, "there are limits to how well an organization can study itself", the report says. "Because the Department's role is to promote energy production, there is an inherent potential conflict between immediate production goals and health and safety goals".

Descriptive epidemiology "describes trends of risk and disease", according to the report. Such work simply describes who is getting diseases, where the diseases are most common and what may cause them. Because the work is based on straightforward statistics, the advisory panel concluded that DOE would not face a substantial credibility problem if it continued to oversee that portion of the epidemiology programme. Watkins said the report was "exactly what I wanted" and he would accept the recommendations.

G. Christopher Anderson

PHARMACEUTICAL PATENTS

Europe to close gap?

London

NEW regulations to bring patent protection for pharmaceutical products in European Communities (EC) countries in line with the United States and Japan were launched by the European Commission last week.

Drug companies file for patents before clinical trials. But drugs are licensed only after exhaustive testing, which means that a standard 20-year patent typically has only eight years to run by the time a drug comes onto the market. The new regulations will give certificates to extend patent protection up to a maximum of 16 years from a drug's commercial launch, similar to schemes adopted recently in the United States and Japan.

If approved by ministers from EC member states, the regulations will come into force immediately, overruling national legislation. The Association of the British Pharmaceutical Industry is now urging the British government to support the proposed regulations, placing press advertisements to highlight its case.

Peter Aldhous

GENETIC ENGINEERING

Transfer study expands

Washington

A SUBCOMMITTEE of the US Recombinant DNA Advisory Committee (RAC) lifted restrictions on the number of patients for a key gene transfer trial last week, but postponed a decision on an application for the first gene therapy experiment.

At the meeting of a subcommittee of RAC, members voted unanimously to lift the cap on patient numbers, now set at ten, for the continuing gene transfer study of patients with widespread melanoma. Although, in principle, the patient-number limitation has been removed, the Institutional Biosafety Committee (IBC) of the National Institutes of Health (NIH) has requested a temporary cap of 50 patients.

The study, sanctioned in January 1989 after a lengthy federal review process, is a joint effort between W. French Anderson of the Heart, Lung and Blood Institute and R. Michael Blaese and Steven A. Rosenberg of the National Cancer Institute (NCI). It involves readministering interleukin-2-stimulated T-lymphocytes marked with the gene for neomycin resistance to patients from whom the cells were isolated. Although the gene confers no

therapeutic benefit, it allows the researchers to track the cancer-killing tumour-infiltrating lymphocytes (TIL) in the patient's body. So far, six of the ten patients have been treated, with data on the first five submitted to the *New England Journal of Medicine*. The TIL appear to 'home' to the tumour site, but whether this correlates with anti-tumour activity has yet to be determined.

Now that researchers have established that the gene transfer protocol is safe, they hope to introduce genes of therapeutic value, such as tumour necrosis factor and interferon. Rosenberg will be submitting the next phase of the TIL protocol for review within two months, but because the subcommittee and the RAC meet infrequently the protocol will be subject to delays of six months or more.

No decision was reached by the subcommittee in its review of the first human gene therapy protocol, which is proposed by Blaese. It is designed to treat ADA (adenosine deaminase) deficiency — a rare inherited immune disorder affecting fewer than 20 people worldwide. Lack of a functional ADA gene accounts for about

UK RESEARCH FUNDING

Polytechnics still persecuted?

London

BRITISH universities and polytechnics have diverged in their responses to a Department of Education and Science (DES) consultative paper which suggests changes in the method of funding for British scientific research.

The paper proposes reform of the 'dual support' system, whereby the universities receive nearly £620 million a year from the Universities Funding Council (UFC) to cover research costs, in addition to research council grants. The government argues that the distinction between universities' and research councils' funding responsibilities has become increasingly blurred, and propose a transfer of £70 million a year to the research councils from 1991–92 (see *Nature* 343, 199; 18 January 1990). Research council grants will then include a component for overheads.

The dual support system has never embraced the polytechnics, which have no large research component in their block grants from the Polytechnics and Colleges Funding Council (PCFC). Understandably, the Committee of Directors of Polytechnics (CDP)'s view is that the proposals are merely "a very cautious tinkering" with an unjust system. One delegate at a CDP conference, called to discuss the proposals, described the present situation as a "dramatic intellectual apartheid".

The CDP is annoyed that the consul-

tative paper proposes a shift of some of the PCFC's minimal research support to the research councils, while leaving most of the UFC's research money untouched. It suggests a larger transfer of UFC funds (so that research council grants would cover all the costs associated with research projects, including academic salaries), to enable the polytechnics to compete equally.

This view was pressed strongly by Professor Terence Burlin, rector of the Polytechnic of Central London, at the CDP conference. He asserted that, with the exception of medical, dental and veterinary schools, universities have no additional costs that merit the continuance of the dual support system.

For the universities, the Committee of Vice-Chancellors and Principals (CVCP) says that any transfer of UFC money may threaten their research. The CVCP argues that difficulties with the present system lie in underfunding, not in the balance of responsibility between universities and research councils.

But Sir Graham Hills, principal and vice-chancellor of the University of Strathclyde, believes the government's planned expansion of higher education is possible only if teaching and research are costed separately, and one approach would be to dismantle the dual support system.

Peter Aldhous

SCIENTIFIC PUBLISHING

Indians say give us Nature!

New Delhi

EIGHTY-three per cent of Indian scientists polled recently want the government to rescind a 50-year-old policy that prevents foreign publishers of scientific journals from bringing out Indian editions. And more than 90 per cent of would like to see Indian editions of *Nature*, *Science* and *Naturwissenschaften*, while two-thirds want local editions of *New Scientist*, *Scientific American* and *La Recherche* as well.

The opinion poll was conducted by *Current Science*, a journal of the Indian science academy, at the request of the Department of Science and Technology (DST). DST is reviewing the existing ban on foreign publications, introduced in the early 1950s out of fears that allowing Indian editions of foreign journals would stifle the growth of local science journals (see *Nature* 341, 557; 1989).

K.S. Jayaraman

25 per cent of children who have severe combined immunodeficiency syndrome (SCID). Children with SCID due to ADA deficiency can be cured in 30 per cent of cases where a suitable sibling-matched bone-marrow donor can be found.

Absence of a functional ADA gene results in the build-up of a toxic chemical that destroys the immune system. Blaese hopes to correct the ADA deficiency by inserting a normal ADA gene into T cells recovered from the patient using the same transfer methods used in the TIL protocol.

But the review coincidentally came in the same week as the US Food and Drug Administration (FDA) approved the use of polyethylene glycol-conjugated bovine ADA (PEG-ADA) as another treatment for ADA deficiency. With two potential therapies for ADA deficiency, recruitment of patients could become more difficult, says Abbey Myers of the National Organisation for Rare Disorders, a subcommittee member.

Blaese said that "there is no question that patients are getting better on PEG-ADA, but the real issue is can we do better for them?". In the protocol guidelines, it is envisaged that most of the patients will be receiving PEG-ADA as well as gene therapy. PEG-ADA will be withdrawn only when the ADA gene-modified T cells have been shown to be effective by themselves.

The proposal must wait for further scrutiny by the subcommittee on 1 June, then go to the full RAC in October for final approval.

Henry I. Miller of the FDA expressed concern that the long delays in the review process caused by "gratuitous over-regulation" will serve as a "disincentive to carry out research and development".

Diane Gershon

Being black in South Africa

SIR—G. Osthoff (*Nature* 343, 110; 1990) wishes to convince us that the educational prospects of South African non-whites are not disadvantaged by the political system they live in, but are disadvantaged by their culture. Moreover, he wishes to attribute similar causes to the lower levels of black American enrolment in higher education in the United States partly by raising the issues together.

Leaving aside the different motivations of different populations and the priority given to higher education in different communities, his thesis represents a popular position among Afrikaans and their English-speaking apologists in justifying the current allocation of funds to the education of blacks in South Africa. This thesis has many defects. First, South African tribal blacks are introduced to English and Afrikaans at a relatively advanced age so they must make a linguistic reorientation as their later tuition is not done in their native languages. Separation of schools could be justified on a linguistic basis but the decision to institute a linguistic reorientation cannot be a cultural one. Furthermore, no native-born white child in South Africa has this limitation. A curious irony of this system is that Afrikaner children were considered to be the class dunces before Afrikaner nationalism because a powerful political force and when Afrikaans and, earlier, Dutch were forbidden in classrooms. Memories of such treatment still provide much bitterness among Afrikaners.

Second, South African blacks are technically not citizens and so are sequestered to 'homelands'. Moreover, they may not live where they choose nor can they own property, and they are unlikely to earn sufficient money to be able to send their children to the schools of their own choosing. Again, these are not cultural aspects of their lives but constitute part of the legislation of South Africa.

Third, most South African blacks do not pay much tax because they do not earn much money: they are not paid equal wages for equal work. Indeed, they cannot rise to the level of their own competence because they are generally not hired under principles of equal opportunity for work which is more than semi-skilled. Under these circumstances, what incentive is there for black South Africans to go to university to study mathematics when they would have little or no expectation of being employed? Most are so affected by the hopelessness of their political situation that to spend three or more years at a university instead of trying to earn what meagre wage they can to survive is seen as a gross waste of time.

My own experience of South Africa is not that of a journalist, visitor or immi-

grant, but that of a South African who went through the non-white educational system. It was only by emigrating from South Africa that I could pursue a professional career in science.

Finally, does anyone see the relevance of the kinds of sport that South Africa blacks pursue and what relevance does all this have for black Americans?

WILLIAM BARENDSE

*CSIRO Tropical Cattle Research Centre,
Box 5545,
Rockhampton Mail Centre,
Queensland 4702, Australia*

SIR—Benatar's Commentary on a selective academic boycott of South Africa (*Nature* 343, 505; 1990) could hardly have appeared at a better time. The recent changes in the country are enough to reopen this question among even the most determined advocates of the boycott, but the opportunity for some relaxation had already appeared. The African National Congress has approved of the Union of Democratic University Staff Associations (UDUSA) and has itself resolved "to encourage and promote academic exchanges to advance scholarship in teaching and research, provided these exchanges are consistent with . . . non-racism and opposition to apartheid . . . reserving the right to protest against such exchanges which have the effect of legitimating and strengthening the apartheid system". This policy of selective support for academic exchanges is now more relevant than ever.

I have personally always opposed the academic boycott, which I believe has done more harm than good (in which respect I have always made my opposition to the policy of the Association of University Teachers quite clear to my colleagues on the executive committee). I understand that the former South African AUT (located in the 'open' universities) has now merged with the UDASA, as has the Witwatersrand Staff Association. I am glad to say that the AUT has now recognized UDASA. It follows from this that the academic boycott should be lifted, subject to the qualifications of the ANC.

JOHN GILLARD WATSON

*11 Beaumont Buildings,
Oxford OX1 2LL, UK*

Letters from America

SIR—The interpretation put upon the origin by country of letters published in *Nature* by J. F. Lamb (343, 404; 1990) is only one of several. Could we not equally surmise that the recent increase in letters from the United States may reflect better

science now emanating from that area, rather than a worsening of that in the United Kingdom? As current figures reflect population numbers, should we not rather congratulate ourselves that lower levels of support here produce science that is equal to that of the United States, as judged by the *Nature* criterion?

Further, the sacred spectre of editorial judgement has not yet been raised. Could the opening of the Japan office of *Nature* have anything to do with the increase in that country's representation? Assuming only fine differences exist between some papers that are accepted or rejected, perhaps in those cases editorial decisions are inevitably influenced by country of origin? Could there even be a quota, unconscious or otherwise? Finally, does the huge increase in physics papers from West Germany, unremarked by the author, reflect increased funding levels by that country compared to others? If so, this might be a better illustration of a direct correlation between funding and publication in *Nature*.

P. J. BARNARD

*MRC Molecular Neurobiology Unit,
Hills Road, Cambridge CB2 2QH, UK*

Schrödinger

SIR—May I add some information about Schrödinger (see *Nature* 341, 193, 1989; 337; 1989 and 343, 686; 1990)? On the occasion of Schrödinger's seventieth birthday in 1957, I (then Science Editor) wrote an article about his work and life for the Viennese newspaper *Arbeiter-Zeitung* after interviewing him at his home. As I wanted to be sure that my article was correct, I sent him the manuscript. He made some corrections which he explained in his covering letter of 24 July 1957 (which I donated to the Austrian National Library) and wrote also:

"... I would like very much to have the word Nazi opponent [which I had used in my article] replaced by a better one. For I am very unpolitical. (*Sehr gerne hätte ich das Wort Nazigegner durch ein besseres ersetzt. Ich bin nämlich sehr unpolitisch* — Schrödinger's emphasis) Nevertheless, I am for example 'an opponent' of assassins and sex murderers, sadists and murderers and arsonists. I remember clearly my three years in England (1933–36), when people listened to me willingly. But I felt that they thought in their hearts: 'Oh well, he is just from the other party, therefore, he rails. Would perhaps democrat or liberal do . . . ?'"

At the end of the letter he expressed his veiled sympathy for the Austrian Social Democrats of which *Arbeiter-Zeitung* was the party newspaper:

"... With a few small alterations it will be a nice birthday greeting with which I will be cordially pleased, from a side which among all [the last two words added later] in any case is nearest to me."

FRIEDRICH KATSCHER

*Mariahilfer Strasse 133,
A-1150 Vienna, Austria*

Towards explaining superconductivity

The high- T_c superconductors contain planar sheets of copper atoms, structures on which much attention is being lavished — with some success.

ALTHOUGH it is curious that, almost exactly three years after the discovery of the first ceramic material capable of supporting superconductivity above the temperature of liquid nitrogen, there is still nothing that might be called a simple theory of the phenomenon, at least it becomes progressively easier to understand why that should be so. At first, of course, it seemed that the materials found to be superconducting have a common structural element that would quickly lead to an explanation of their properties. The working elements are two-dimensional sheets in which copper ions of ambiguous valency are linked to others through oxygens. What more reasonable than to suppose that the problem is that of accounting for conduction by electrons in these copper sheets, on the assumption that the function of the other components of the copper oxide superconductors is simply to hold the working sheets together, and in the process to modulate their electrical properties?

The guess may be correct, but the inference is not especially helpful. The copper ions in superconducting sheets do not all have complete shells of electrons, and so have magnetic moments instead. Although the sheets of copper atoms in a compound such as La_2CuO_4 — from which superconductors may be derived by replacing some La by Sr for example — may be regarded as simple prototypes for the superconducting structures, this compound is itself an insulator which, at low temperature, has the properties of an antiferromagnet — there is long-range magnetic ordering of the magnetic ions, but neighbouring spins tend to point in opposite directions, so that there is no overall magnetic moment.

By now, it seems to be generally accepted that most of the high-temperature superconductors are converted, by relatively small changes of chemical composition, into materials with long-range antiferromagnetic order (but no superconductivity) at low temperature. But the measurable order of the copper ions can be taken as a proxy for the ordering of whatever electrons they may have released into the electron bands of the lattice. So the problem of accounting for the electrical properties of the superconducting copper sheets may be likened to that of accounting for the properties of a two-dimensional lattice in which there are spins at all (or almost all) the lattice points, and in which the interaction between

them predisposes towards antiferromagnetism — the energy of two parallel neighbouring spins is greater than that of two anti-parallel spins at the same location.

If the spins were those of classical objects, there would be no difficulty. Antiferromagnetism on a two-dimensional lattice would be no different from, say, the problem of filling a lattice with equal numbers of atoms of two different kinds, which is the two-dimensional equivalent of the order-disorder transitions found in, for example beta (GK beta)-brass. But, sadly, electrons being electrons, the problem is not classical but quantal, and the problem is no less difficult than that of enumerating the states of a system of spin- $1/2$ quantum spins at the vertices of a two-dimensional lattice. Endless assaults on the problem of ferromagnetism have shown how difficult this task can be.

The essence of the difficulty is that, while it may be possible to guess (and even prove) that the ground state of the whole system (and thus that occupied at sufficiently low temperature) may be a ferromagnet or antiferromagnet according to the direction of the coupling feedback, even the low-temperature thermodynamic properties of the system depend on the numbers of states of not much higher energy accessible to the system. The obvious candidates are states in which patches of order are separated by domain boundaries, but enumerating them is not child's play.

Yet some progress seems to have been made. Thus Hong-Qiang Ding and Miloje S. Makivić, from California Institute of Technology, now describe an exceedingly powerful Monte Carlo calculation of an antiferromagnetic lattice designed to allow for the simulation of La_2CuO_4 (*Phys. Rev. Lett.* **64**, 1,449; 1990). In this context, a Monte Carlo simulation entails starting with an arbitrary arrangement of spins on the lattice, and then changing them in pairs according to rules that allow all spin states to be reached without violating the overall constraints. The authors rightly boast of their access to Caltech's parallel computer system, but they have also devised a new and efficient algorithm for tracing out the evolution of their system. As is the custom in this part of the trade, they have worked with square patches of two-dimensional lattice with as many as 128 lattice spacings to each side.

The outcome is a relationship between correlation length — the distance over

which order, on the average, persists — and temperature; briefly, the logarithm of the correlation length is inversely proportional to the temperature. That, apparently, contradicts other models of the ordering process. In lanthanum copper oxide, the correlation length agrees well with that measured by neutron diffraction below 500 K (where there is a phase transition), provided the interaction energy is chosen appropriately. For what it is worth, that energy is not very different from estimates derived from Raman-scattering experiments, which provide a direct measurement of the energy of interaction by the change of frequency of the scattered light.

All that is encouraging enough, but not every calculation in the field is like that. The same issue of the same journal (**64**, 1445; 1990) describes a different approach to the same problem by H. J. Schulz of the Université Paris-Sud. The starting-point is again an arrangement much like that of lanthanum copper oxide, but the objective is to understand what happens as electrons are added to or subtracted from the system by the substitution of lanthanum atoms by others. The conclusion seems to be that the detailed structure of the magnetic ordering is exquisitely sensitive to the addition or subtraction of electrons from the lattice.

Imbalancing the lattice in one direction or the other will stimulate the formation of domain walls, at decreasing separation from each other as the imbalance grows. Indeed, Schulz suspects that there is no composition except that corresponding to stoichiometrically pure lanthanum copper oxide over which a single antiferromagnetic domain accounts for the whole of the structure. In this picture, the emergence of a conducting, let alone a superconducting, state seems obscure, to say the least.

There is no inconsistency between these approaches, which are complementary to each other. The more frequently domain walls appear in the structure, the less the long-range order and thus the correlation length. But no doubt it is now only a matter of time — time on Caltech's parallel computer — before there is a sequence of calculations at various degrees of imbalance from the model equivalent of lanthanum copper oxide. It will be interesting to see what emerges, but even success will not necessarily imply that there will be an explanation to put in the textbooks.

John Maddox

Switching arrays make light work in a simple processor

David Brady

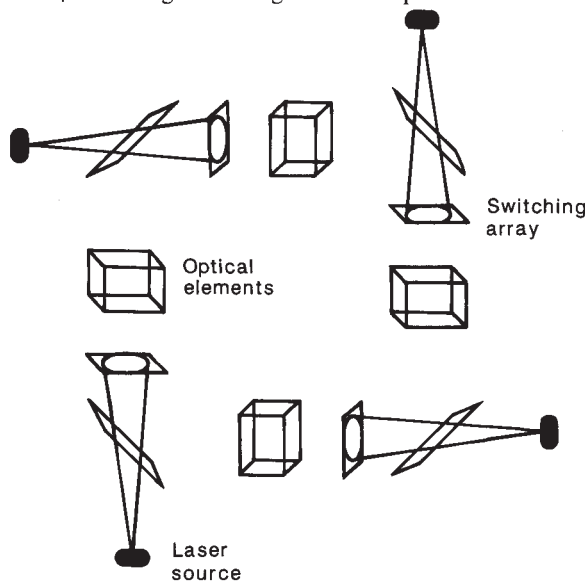
THE announcement at a recent meeting* of the demonstration of a small digital optical information processor — the first step to an optical computer — was accompanied by great public fanfare (see, for example, refs 1, 2). But despite the apparent attractions of optical computation — speed, parallelism and good isolation of signals from interference, for example — it is not yet clear whether it can supersede electronic methods.

The device, built at AT&T Bell Laboratories, consists of four planar arrays of 32 optical switches (H.S. Hinton and M. Prise). Information is transmitted from one array to the next by light signals propagating perpendicular to the surface of the planes (see figure). Light passing through the fourth array is redirected onto the first to form a closed processing loop. Each optical switch consists of two input ports and an output port. The signal incident on a control input port determines the connection between the other input port and the output port.

In the processor, the output of a switch on the preceding array enters the control port of a given switch and determines its output. The other input port is provided with a strong constant signal. If the control signal is above threshold then the constant input is connected to the output and a strong output signal is generated. If the control input is below threshold, a much weaker output results. Although the physical structure of the optical switch used by the AT&T group — the symmetric self electro-optic effect device, or S-SEED — is substantially more complex than a standard electronic transistor switch, the basic function is the same.

The nature of the computation performed by the processor is determined by the connections between switches. These connections are specified by optical elements, such as lenses, mirrors and masks, which control the passage of light from one plane to the next. The AT&T group selected these elements to shift the outputs of the switches on one array onto the control inputs of the switches on the next array to run the processor as a counter, a system that cycles through a periodic series of states. Although the capability of the processor itself is not remarkable,

the experiment is of interest as a demonstration of the ability of optical switches to operate in digital systems. Specifically, it shows that digital optical switches can be cascaded and operated in parallel arrays. The S-SEED is a sophisticated descendant of a class of switching devices developed over the past 15 years by groups at Heriot-Watt University³ and the University of Arizona⁴. Although, if the popular press is to be believed⁵, the Arizona group has become actively hostile to optical computing, the group at Heriot-Watt has demonstrated cascaded switching in a single-channel processor⁶



Schematic layout of the AT&T optical processor. The processor consists of four arms, each containing a laser source, a switching array and interconnecting optical elements. The output of each arm is fed into the control inputs of the next by a half-silvered mirror. The laser sources clock the propagation of signals around the loop and generate the constant inputs. The processor is programmed by physically specifying the optical elements.

and is currently working on systems similar to the AT&T group's (B.S. Wherrett).

To understand the significance of these experiments, one must consider the motivation for constructing optical computers. Electronic charges play a role in all practical switching technologies. An optical switch differs from electronic switches in that photon-electron interactions play a role, often in addition to conventional electronic interactions. (The S-SEED is a hybrid 'photonic' and electronic device. The devices used by the Heriot-Watt group are more nearly purely optical.) Speed, size and the ability to communicate are among the most important attributes of a digital switch. Because the operation of a switch always involves

the transport of a charge proportional to the device's size, its speed is generally linearly related to its size. Size also limits the number of devices that can be constructed on a plane and thus incorporated into the system. But because the wavelength of an electron is smaller than the wavelength of light, the fundamental advantage of small size, and thus of high speed and device number, belongs to purely electronic systems. (Statistical considerations and fabrication processes limit the size of electronic devices well before the electron wavelength is reached.)

Optical switches are potentially superior, however, in the area of communications. The advantage lies not in the erroneous proclamation in news reports on AT&T's work that electronic signals move at a slower velocity than the vaunted speed of light. (The spirit of the phrase "speed of light" seems to add an unwarranted cachet to optical processors.) Nor is it really advantageous that an optical signal can be sent through free space, whereas a wire is needed to convey an electronic signal, although this is closer to the point. The basic problems for electronics are the awkwardness of two-dimensional wiring between components, the difficulty of isolating electronic signals over a wide bandwidth (high information throughput), and the fact that the energy lost in communicating between electronic devices can exceed that needed to switch successive devices. The first problem arises in processors that cannot be laid out on a plane such that each switch is connected only to near neighbours. The second arises when signals must be transmitted over some distance and accounts for the popularity of optical-fibre communications systems. The third problem arises from the relatively 'lossy' nature of communications in integrated electronic devices.

The use of optics to solve the third problem has been termed 'quantum impedance conversion' by David Miller of AT&T⁶.

Although the qualitative basis of the communications problem for electronic circuits is understood, the quantitative evaluation of whether and how a given architecture would be improved by optical communications is difficult⁷. Certainly, fibre communication lines are an improvement on other methods in very high-bandwidth applications and, just as certainly, simple electronic chips are not going to be improved by substituting optical versions. The usefulness of optical communications between digital switches remains an open question. As with other developing technologies, the question

* SPIE Conference on Digital Optical Computing, Los Angeles, January 1990.

hanging over digital optical work is "Can you build something useful better with methods other than straight electronics?". Although the experiments at AT&T and Heriot-Watt answer the question "Can you build something?" in the affirmative, the larger question remains unanswered. Unfortunately, the advantage of optics — communications capability — can only be demonstrated in large-scale systems.

When the transistor was invented, people knew immediately that something useful was afoot. With optical switches, one cannot know for sure until a substantial network of switches is grinding away. The latest demonstrations certainly strengthen the feeling that something use-

ful will come of communications through arrays of optical switches, but this step does not seem to have come easily and it is still a long walk to a general-purpose digital optical computer. □

David Brady is in the Department of Electrical and Computer Engineering, University of Illinois, Urbana-Champaign, Illinois 61807, USA.

1. Markoff, J. *New York Times* 1 (30 January, 1990).
2. Hooper, L. & Schelesinger, J.M. *Wall Street Journal* 1 (30 January, 1990).
3. Smith, S.D. *Nature* **316**, 319–324 (1985).
4. Gibbs, H.M. *Optical Bistability: Controlling Light with Light* (Academic, New York, 1985).
5. Smith, S.D., Walker, A.C., Tooley, P. & Wherrett, B.S. *Nature* **325**, 27–31 (1987).
6. Miller, D.A.B. *Opt. Lett.* **14**, 146–149 (1989).
7. Goodman, J.W., Leonberger, F., Kung, S. & Athale, R.A. *Proc. IEEE* **72**, 850–859 (1984).

HYPERTENSION

Stress on the renin gene

Brenda Leckie

A NEW twist to an old story¹ appears on page 541 of this issue, in a report by Mullins, Peters and Ganten² who show that transgenic rats bearing an extra gene for renin develop high blood pressure. At first sight this is not surprising because it is well known that injection of renin into an animal causes a rise in blood pressure. The most interesting point is that the rats are hypertensive although concentrations of renin in the kidney and plasma are low.

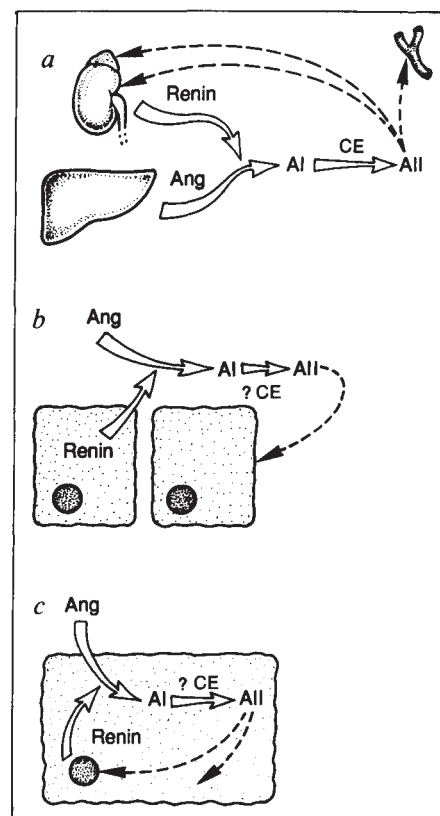
In the classical scheme, renin is synthesized by the kidney and secreted into the blood, where it generates the pressor hormone angiotensin II (see figure). But most patients with high blood pressure have normal or low circulating concentrations of renin and angiotensin II. What, then, causes blood pressure to rise excessively in some individuals? There is a genetic component, in that the children of hypertensives also tend to develop hypertension. Genetically hypertensive strains of rat have been bred, but the genes responsible for the hypertension, and its physiological basis, are obscure. Environmental factors also play a part, but experts differ as to what these are. Excess sodium chloride in the diet, and stress, have both been implicated. But how much salt is too much? And is it more stressful to be chased by a sabre-toothed tiger or to drive on the M25 motorway?

The production of transgenic animals is a step towards understanding the interactions between genes and environment that result in hypertension. The rats were transgenic for the *Ren-2* gene, which is expressed in mouse salivary glands, whereas the *Ren-1* gene is expressed in kidney¹. Only the *Ren-1* gene is normally present in rats and humans. The structure and activity of the enzymes encoded by *Ren-1* and *Ren-2* are very similar, but the gene sequences diverge at a point 179 base

pairs upstream from the transcription start site³. The transgenic insert contained 5.3 kilobases of the 5' end, so it contained the controlling sequences present in *Ren-2* of the mouse. Interestingly, it was expressed in the adrenal glands rather than in the kidney. Unlike kidney renin, *Ren-2* tissue expression in mouse salivary gland is enhanced by androgens⁴, but both male and female transgenic rats expressed the gene and were hypertensive, suggesting that androgens were not controlling the expression of *Ren-2* in their adrenals.

The transgenic model described by Mullins and colleagues has certain similarities with human essential hypertension: blood pressure increases as the animals mature, and concentrations of active renin in the plasma are normal or low. However, there are differences. In humans, overt hypertension develops after puberty, although 'tracking' of blood pressure towards a higher level is apparent in childhood⁵. If renin and blood pressure are measured in a human population, normal distributions for these parameters are obtained, as expected for a polygenic disorder. In contrast the transgenic animals were either clearly hypertensive or clearly normotensive.

Perhaps the origin of the hypertension in the transgenic rats is more akin to that of the rarer forms of human hypertension associated with mineralocorticoid abnormalities, rather than to essential hypertension. In the human, adrenal hyperplasia can produce aldosterone-dependent hypertension. An even rarer 'dexamethasone-suppressible hyperaldosteronism' is inherited as an autosomal dominant disorder. A link between renin and adrenal function has not been demonstrated in these disorders, but it is possible that expression of renin in the adrenal cell could lead to high local concentrations of



a, The classical scheme: renin is released by the kidney and acts on its substrate angiotensinogen (Ang), which is secreted by the liver, to produce the decapeptide angiotensin I (AI). This in turn is split by converting enzyme (CE) to give angiotensin II (AII). AII contracts vascular smooth muscle and promotes its growth; it stimulates aldosterone secretion by the adrenal; and it promotes sympathetic transmission at the synapse. b, Scheme for local production of AII within the interstitium of the adrenal gland. c, Scheme for intracellular production of AII. Ang would have to be produced by, or enter, the cell, and CE would have to be present.

angiotensin II in the interstitial fluid (see figure, b) or within the cell (c). This might stimulate cell growth, overproduction of aldosterone with associated sodium retention, and suppression of both renal and plasma renin concentrations.

Oversecretion of aldosterone is not characteristic of human essential hypertension⁶, nor do individual patients show sodium retention⁶ or gross abnormalities of glucocorticoid production. Slight abnormalities of glucocorticoid production in early life might be implicated in the

1. Mullins, J. *et al. EMBO J.* **1**, 1461–1466 (1982).
2. Mullins, J.J., Peters, J. & Ganten, D. *Nature* **344**, 541–544 (1990).
3. Panthier, J.J., Dreyfus, M., Tronik-Le Roux, D. & Rougeon, F. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5489 (1984).
4. Wilson, C.M., Erdos, E.G., Dunn, J.F. & Wilson, J.D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1185–1189 (1977).
5. Munger, R.G., Prineas, R.J. & Gomez-Martin, O.J. *Hypertension* **6**, 647–653 (1988).
6. Beretta-Piccoli, C. *et al. Clin. Sci.* **63**, 257–270 (1982).
7. Naruse, M., Sussman, C.R., Naruse, K., Jackson, R.V. & Inagami, T. *J. clin. Endocrinol. Metab.* **57**, 482 (1983).
8. Baba, K., Mulrow, P.J., Franco-Saenz, R. & Rapp, J.P. *Hypertension* **8**, 1149–1153 (1986).
9. Wang, S.-M. & Rapp, J.P. *Molec. Endocrinol.* **3**, 288–294 (1989).
10. Lever, A.F. *J. clin. Hypertension* **3**, 323–327 (1987).

later development of hypertension, but the link between adrenal renin and steroids is still obscure. Low levels of renin are present in human⁷ and rat adrenals. Interestingly, there are differences in the levels of adrenal renin in salt-sensitive and salt-resistant genetically hypertensive rats⁸. In these rats, a restriction fragment length polymorphism of the renin gene segregates with hypertension⁹. It would be fascinating if local production of angiotensin II controlled glucocorticoid output in such a way as to induce changes in cellular morphology in key organs such as blood vessels¹⁰, the heart or kidney. The renin gene (*Ren-1* in normal rats and humans)

may, in turn, be regulated by steroids. A negative regulation might mean that the original abnormality was switched off, although the effects persisted.

A number of other candidate genes for hypertension, apart from renin, are being investigated. The future lies in identifying genes that predispose to polygenic disorders such as hypertension and in linking gene expression, which may be influenced by the environment, with the development of the disease. □

Brenda Leckie is in the MRC Blood Pressure Unit, Western Infirmary, Glasgow G11 6NT, UK.

ENZYME SYSTEMS

Better reception for urokinase

Walter F. Mangel

THE fibrinolytic enzymes have been implicated in a wide variety of normal and abnormal physiological processes requiring extracellular proteolysis, including fibrinolysis, cell migration, tissue remodelling and metastasis^{1,2}. The enzyme system consists of the serum zymogen plasminogen, which is activated by cleavage of a single peptide bond by either urokinase or tissue plasminogen activator to give the potent serine proteinase, plasmin. Over the past seven years there has been great interest in the role of tissue plasminogen activator in dissolution of the fibrin clots that cause heart attacks, and the enzyme became the first major new drug of the recombinant DNA industry. There has been renewed interest in urokinase with the discovery that it has its own specific receptor on the cell surface^{3,4}, and now the purification and cloning of this receptor are respectively reported by Behrendt *et al.*⁵ and by Roldan *et al.*⁶

A paradox with the fibrinolytic enzyme system has been that all the components coexist in a large volume — in humans, this corresponds to 5 litres of plasma and extravascular fluids — yet activation of plasminogen occurs only at specific sites. If activation were not restricted in this way, so much plasmin would have to accumulate to dissolve a fibrin clot that we would lyse from within. For tissue plasminogen activator this paradox has been resolved, because when it binds to fibrin the Michaelis constant for the activation of plasminogen decreases by 100-fold, down to the *in vivo* concentration of plasminogen⁷. However, urokinase does not bind to fibrin but appears to be more involved in extracellular proteolysis mediated by cells. With the characterization of the urokinase receptor, the mechanism of targeting urokinase activity should become clearer.

Urokinase activity in cells increases during physiological processes involving

cell migration or tissue remodelling. Urokinase can also cleave fibronectin⁸, and plasmin cleaves proteins of the extracellular matrix and basement membrane and activates the precursor to collagenase⁹. So theoretically, this enzyme system can free a cell from constraints in its immediate environment and remodel that environment. For example, when a confluent monolayer of capillary endothelial cells is wounded by a razor blade, the cells that migrate into the denuded area from the edges increase their urokinase activity, which subsides to normal when movement stops and the wound is closed¹⁰. During mouse embryogenesis, urokinase messenger RNA levels vary greatly¹¹. From fertilization up to day 4 of development, before implantation in the uterus, there is no detectable mRNA. Levels rise in the trophoblasts between days 5 and 7 of gestation, when the uterine wall is penetrated. After a transient reduction, urokinase mRNA synthesis increases in the peripheral giant trophoblast cells that surround the embryo at the three-to-seven somite stage, which is coincident with a period of major embryonic expansion. The biochemical details of how this increase in plasminogen activator is put to use are not yet clear, but the effects are probably mediated by the urokinase receptor.

The importance of the interaction of urokinase with its receptor is illustrated by the reduction in efficiency of invasion of resealed chick embryo chorioallantoic membranes by tumour cells to 25 per cent when urokinase is not bound to its receptor¹².

The urokinase receptor has a dissociation constant of 0.3 nM, and is found on the surface of monocytes at a density of 40,000 receptors per cell, and is also present on many other cell types^{3,4}. Urokinase consists of domains, a growth-factor domain at the amino terminus followed by

a connecting peptide, a kringle and the protease domain. Binding to the receptor is through the growth-factor domain¹³ and occurs by a novel autocrine mechanism, in which a cell secretes urokinase which then binds to the urokinase receptor on its surface¹⁴. Receptors of urokinase are not evenly distributed over the surface of a cell¹⁵. On fibroblasts and fibrosarcoma cells, urokinase is found at discrete cell-substratum contact sites and at areas of cell-cell contact. There is a striking colocalization with strands of vinculin, a component of the focal contact site¹⁶. These are sites where cultured cells adhere extremely closely to the substrate and contain not only extracellular matrix receptors, but also actin, α -actinin and talin¹⁷. Thus, through the urokinase receptor, contact between sites of cell adhesion to substratum and the cytoskeleton may be regulated by urokinase. An antibody against chicken plasminogen activator inhibits overgrowth and degradation of the extracellular matrix as well as the morphological changes associated with transformation of chicken embryo fibroblasts by Rous sarcoma virus¹⁸.

Complementary DNA encoding the urokinase receptor from human cells shares no homology with any gene sequenced so far⁶. The predicted protein is relatively small, containing 313 amino acids. A high proportion of cysteine residues is common to many extracellular regions of receptors, but the pattern of cysteine spacing in the urokinase receptor is not similar to that in other receptors. There is no obvious intracytoplasmic domain: tracts of hydrophobic amino acids are present at both the N and C termini. That this is indeed the gene for the urokinase receptor is confirmed by mouse cells that, when transfected with the human gene, bind human urokinase, whereas the mouse receptor binds only mouse urokinase. Although the molecular weight predicted from the cDNA sequence is about 35,000, purified human urokinase receptor has a molecular weight of 55,000 because of extensive glycosylation⁵.

With the cloning of the gene and the purification of the urokinase receptor, the regulation of receptor synthesis and its function can now be investigated. One

Corrections

■ "Cytoskeletal ups and downs" by Michael Way and Alan Weeds (*Nature* **344**, 292–294; 1990). On page 293, the passage "... and there was a greater than twofold increase in mRNA or protein levels of profilin or actin" should read "... and there was a less than twofold increase..."

■ "Between objectivity and subjectivity" by Carl-Ivar Brändén and T. Alwyn Jones (*Nature* **343**, 687–689; 1990). In some editions the two parts of Fig. 1 — views of part of the electron density for the retinol-binding protein — were transposed: the blue figure should be on the left, the green on the right.

complication is that the urokinase secreted by cells is a zymogen that must be converted to a two-chain active form¹⁹. Plasmin can convert pro-urokinase bound to the receptor to the two-chain form which in turn activates plasminogen while still bound to the receptor. What remains to be discovered is the enzyme that activates

pro-urokinase, the substrates for receptor-bound urokinase and for plasmin, and how the interaction among these components is regulated. □

Walter F. Mangel is in the Biology Department, Brookhaven National Laboratory, Upton, New York 11973, USA.

cation results from the absorption of 35-micrometre photons emitted by dust, followed by radiative de-excitation into the upper level of the transition.

Maser amplification is greatest along radial paths through the circumstellar shell (because the outflow produces greater velocity coherence along such paths). Consequently the strongest maser emission comes from lines of sight that pass close to the central star. This emission is dominated by two peaks, one from the front edge of the OH shell, and one from the back edge (R. S. Booth *et al.*, *Nature* **290**, 382–384; 1981). The radial velocities of these two peaks are positioned symmetrically about the radial velocity of the central star, and their difference gives an estimate of the expansion speed of the OH shell.

Ultimately, mass loss from a Mira strips away its entire envelope, leaving the hot, compact core exposed. This has little immediate effect on the total luminosity of the star; but because its surface area is reduced a millionfold, its surface temperature rises by a factor of 30. The star becomes a hot white dwarf, and starts to emit a powerful flux of ultraviolet photons. The photons ionize and heat the circumstellar shell, converting it into a planetary nebula. At about the same time, the OH maser emission starts to disappear. At first the red-shifted maser emission from the back side of the shell fades away as it becomes obscured by the optically thick ionized gas surrounding the central star. Subsequently, the remaining maser emission disappears as the shell is overrun by ultraviolet radiation and the OH radicals are photo-dissociated.

This intermediate phase, when photo-ionization of the circumstellar gas has started but OH maser emission persists, is short-lived: only four sources are known. It is a critical phase because it provides the link between OH–IR stars (which are not normally surrounded by ionized gas) and planetary nebulae (which do not normally reveal OH maser emission). Shepherd *et al.*'s observations of two of these sources establish for the first time that the OH maser emission comes from a well-defined shell. They show this by resolving the maser emission using the MERLIN very long base-line radio interferometer. They determine both the radius of the OH shell (about 10¹² metres) and its expansion speed (about 17 kilometres per second).

Clearly, if these sources are planetary nebulae in the process of formation, evolutionary effects should be discernible on a time-scale of a few tens of years. Who will chance their arm and predict what these sources might look like in 30 years time? □

A. P. Whitworth is in the Department of Physics, University of Wales, PO Box 913, Cardiff CF1 3TH, UK.

1. Reich, E. in *Molecular Basis of Biological Degradative Processes* (eds Berlin, R. D. *et al.*) 155–169 (Academic, New York, 1978).
2. Dano, K. *et al. Adv. Cancer Res.* **44**, 139–266 (1985).
3. Vassalli, J.-D., Baccino, D. & Belin, D. *J. Cell Biol.* **100**, 86–92 (1985).
4. Stoppelli, M. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 4939–4943 (1985).
5. Behrendt, N. *et al. J. biol. Chem.* (in the press).
6. Roldan, A. L. *et al. EMBO J.* **9**, 467–474 (1990).
7. Hoylaerts, M., Rijken, D.C., Lijnen, H.R. & Collen, D. *J. biol. Chem.* **257**, 2912–2919 (1982).
8. Quigley, J. P., Gold, L.I., Schwimmer, R. & Sullivan, L.M. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2776–2780 (1987).
9. Liotta, L. A. *et al. Cancer Res.* **41**, 4629–4636 (1981).

10. Pepper, M. S., Vassalli, J.-D., Montesano, R. & Orci, L. *J. Cell Biol.* **105**, 2535–2541 (1987).
11. Sappino, A.-P., Huarte, J., Belin, D. & Vassalli, J.-D. *J. Cell Biol.* **109**, 2471–2479 (1989).
12. Ossowski, L. *J. cell. Biol.* **107**, 2437–2445 (1988).
13. Appella, E. *et al. J. biol. Chem.* **262**, 4437–4440 (1987).
14. Stoppelli, M. P. *et al. Cell* **45**, 675–684 (1986).
15. Pollanen, J. *et al. J. cell. Biol.* **104**, 1085–1096 (1987).
16. Hebert, C. A. & Baker, J. B. *J. Cell Biol.* **106**, 1241–1247 (1988).
17. Burridge, K. *Cancer Rev.* **4**, 18–78 (1986).
18. Sullivan, L. M. & Quigley, J. P. *Cell* **45**, 905–915 (1986).
19. Petersen, L. C. *et al. J. biol. Chem.* **263**, 11189–11195 (1988).

ASTRONOMY

Removing the seventh veil?

A. P. Whitworth

FEW astronomers would argue with the thesis that many (but probably not all) planetary nebulae are the end-product of an evolutionary sequence in which a Mira star develops into an OH–IR star, and then transforms itself into a planetary nebula. But this thesis can only be established beyond doubt when the short-lived intermediate stage which links OH–IR stars and planetary nebulae has been confirmed by observation. Observations of hydroxyl (OH) maser emission reported by Shepherd *et al.* on page 522 of this issue (*Nature* **344**, 522–524; 1990) appear to provide this confirmation: they unambiguously record the final throes of two OH–IR stars as they cast off the last layers of their diffuse envelopes and stand revealed as naked white dwarves, emitting the ultraviolet photons which will ionize the surrounding circumstellar gas to produce a planetary nebula.

Mira stars are giant stars with masses comparable to that of the Sun, but with radii at least a hundred times larger and average luminosities many thousands of times greater. The luminosity of a Mira varies with a period in the range 100 to 2,000 days, as the star expands and cools, then contracts and warms up again. Miras lose mass at rates in the range 10^{–7} to 10^{–4} solar masses per year. The Sun is probably destined to become a Mira star in another 5,000 million years or so.

The properties of Mira stars are correlated: more luminous Miras tend to have lower surface temperatures, longer periods, faster outflows and higher associated mass-loss rates. These correlations are readily explained. As a giant's envelope swells, its natural period of pulsation increases. At the same time its luminosity increases and its surface temperature falls. This facilitates the conden-

sation of small solid particles ('dust grains') in the outer layers of the atmosphere. The increased luminosity, combined with the increased opacity due to the dust grains, produces an increase in the force of radiation pressure pushing the atmosphere outwards. At the same time the swelling of the star reduces the restraining force of gravity. The upshot is an increase in the rate of mass loss, leading to the 'superwind' phase of the star's evolution (see last week's News and Views article by Charles Alcock, *Nature* **344**, 381–382; 1990).

The observational picture is confused by the fact that some of the differences between Miras are due to differences in initial stellar mass and elemental composition. Nonetheless, the observational evidence suggests that a Mira swells up with time, and that as it does so its luminosity increases, its surface temperature falls, its period increases, and its mass-loss rate grows until the entire envelope is stripped off. Hence the high-luminosity, long-period Miras are thought to be the older ones, about to form planetary nebulae.

The most obvious result of mass loss from a Mira is the build-up of an expanding circumstellar shell of gas and dust. The dust eventually obscures the star at optical wavelengths. The star's luminosity is then absorbed by the dust and re-radiated in the infrared. Thus longer period (older?) Miras are strong infrared sources.

Another consequence of mass loss from a Mira is the development of hydroxyl maser emission at 1,612 megahertz. The hydroxyl radical, OH, is produced by photodissociation of water molecules. The 1,612-megahertz transition is between hyperfine levels of the rotational and vibrational ground state. The population inversion required for maser ampli-

Laser-cooled atoms cooperate

Christopher Foot and Andrew Steane

RECENT striking observations on clouds of very low-temperature caesium atoms trapped by radiation pressure (T. Walker, D. Sesko & C. Wieman, *Phys. Rev. Lett.* **64**, 408–411; 1990) show that, at high densities, the atoms no longer move independently of one another but behave collectively. Under certain conditions this causes the shape of the cloud to change from a simple spherical distribution to a ring with a small ball at the centre. The authors show that this collective behaviour arises from the scattering of photons within the cloud which produces a long-range repulsive force between the atoms. Previously this force has been considered important only for the interior of stars. The collective effect in neutral atom traps is reminiscent of the crystallization of laser-cooled ions trapped by external electromagnetic fields (R. Blümel *et al.* *Nature* **334**, 309–313; 1988). However, for ions, strong electrostatic repulsion is the dominant force and the detailed physics is different from that in neutral atom traps.

The new techniques of laser-cooling and trapping use the radiation force exerted on atoms by absorption and scattering of photons from a counterpropagating laser beam to slow atoms in an atomic beam. The kinetic energy of the atoms is reduced to the equivalent of less than 1 K, so that they can be trapped by the weak magnetic or radiation forces to which neutral atoms are susceptible. In contrast the electromagnetic forces on ions are much larger and so the ions can be trapped at room temperature, or

above, and then laser cooled while in the ion trap.

The trap used by Walker *et al.* was a magneto-optical trap of the type developed by E.L. Raab *et al.* (*Phys. Rev. Lett.* **59**, 2631–2634; 1987). Six laser beams propagate along the positive and negative x , y and z directions, intersecting at the centre of an ultra-high vacuum chamber (Fig. 1). There is also an inhomogeneous magnetic field which is zero at the centre of the region of intersection. In this arrangement, an atom at rest at the centre of the trap experiences radiation forces of the same magnitude from each laser beam: there is no resultant force because the forces from the counter-propagating beams along each axis cancel. However, at any other point the shift in the atomic energy levels caused by the magnetic field (the Zeeman effect) leads to an imbalance and produces a resultant radiation force which pushes the atom towards the centre. The magnetic field is not strong enough for its direct force to be important, so that the device is not a magnetic trap.

Another feature of this arrangement of laser beams is that there is a damping force on moving atoms which arises from the radiation force imbalance produced by the Doppler effect. Under the influence of this damping force, atoms move as if they were in a viscous fluid, so that the effect has become known as 'optical molasses'. In short, the overall operation of the trap is to capture atoms with velocities of a few metres per second and slow them down to a few centimetres per second while they spiral in towards the centre to accumulate in a small cloud of diameter less than 1 mm. The final temperature is close to the Doppler cooling limit, $kT = \frac{1}{2}h\gamma$, where γ is the natural width of the absorbing atomic transition; k and h are Boltzmann's and Planck's constants, respectively. For alk-

ali metals like caesium, this corresponds to a temperature of less than 10^{-3} K.

Walker *et al.* used trapping beams that were spatially filtered, collimated and carefully aligned with respect to the point of zero magnetic field so that the trap potential was particularly well defined. Under these conditions they observed three distinct types of behaviour in the trap. At low densities the atoms formed a small cloud at the centre of the trap and the size of the cloud remained constant as more atoms were loaded into the trap, as

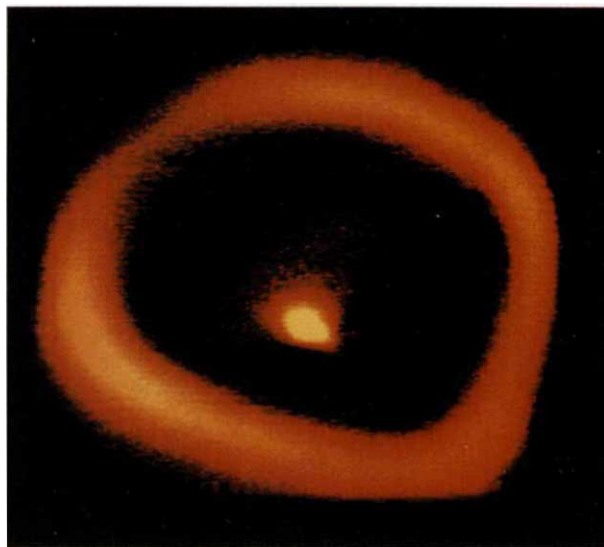


FIG. 2 False-colour pictures of the ring-shaped cloud of caesium atoms observed in a magneto-optical trap by Walker, Sesko and Wieman.

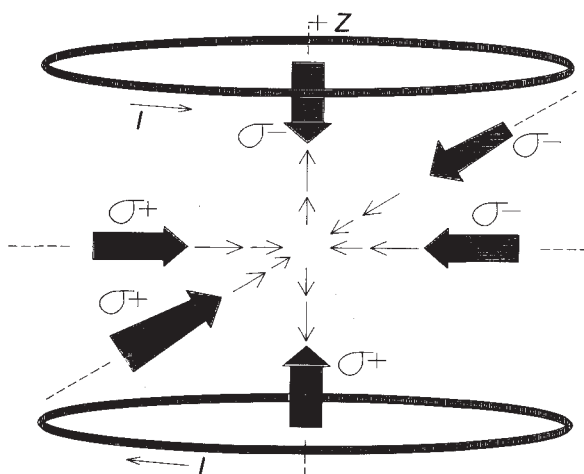


FIG. 1 Magneto-optical trap of Raab *et al.*, in which three orthogonal pairs of counterpropagating laser beams press atoms, whose spectral lines are tuned by an inhomogeneous magnetic field, towards the centre. The 'spherical quadrupole' field is generated by the two current(I)-carrying coils, top and bottom, and lies along each axis as shown by the thin arrows.

expected for a gas of independent particles in a damped harmonic potential. The density within the sphere had a gaussian profile. When the number of trapped atoms increased beyond 40,000, the radiation force between the trapped atoms, from scattering and absorption of photons within the cloud became significant. In this second regime, the atoms had an approximately uniform spherical distribution with constant density, so that as more atoms were trapped the size of the cloud increased. Up to 3×10^8 atoms were trapped in the second regime in a cloud approximately 3 mm in diameter.

In the third regime observed (Fig. 2), there was either a continuous ring or a small orbiting clump about a central ball of atoms. These orbital modes could be produced when there were more than 10^8 atoms and trapping beams were misaligned in one plane by just a few milliradians. The direction of the rotation of the clump of atoms corresponded to the torque produced by the misalignment. Walker *et al.* present a theoretical argument that explains the existence of stable orbits at well-defined radii where the inward trapping force from the laser radiation is balanced by the outward force from photons coming from the central ball of atoms. This can occur if there is a

sufficient number of atoms in the trap, and if the cross-section for absorption of a scattered photon is greater than that for absorption of a laser photon, which was possible because the frequency distribution of scattered light was not the same as that of the laser light.

The observation of collective behaviour in neutral-atom traps at moderate densities is another unexpected and rather beautiful result in this new field.

INSECT FLIGHT

Unconventional aerodynamics

Roland Ennos

MORE evidence has appeared showing that insects fly by mechanisms quite unlike those used by aeroplanes and helicopters. Zanker and Gotz¹⁻³ have measured the instantaneous forces produced by tethered *Drosophila melanogaster* flies and find that they cannot be explained by conventional aerodynamic theory. The forces are also evidence that these flies have unusual methods for producing lift.

During the steady flight of an aircraft, air travels faster over the top of the wings than over the bottom and so there is a net circulation of air around the wings. It is the movement of the wing through the air with its attached circulation that results in lift. But if an aerofoil is accelerated from rest it must move a distance equal to several times its own width before the circulation around it and the lift it produces can build up to the steady-state values, a phenomenon known as the Wagner effect.

Conventional analyses of animal flight treat the wings like those of fixed-wing aircraft; they assume that, at each instant, each wing section will produce the same lift as a wing section moving at the same steady velocity and with the same attitude. Total lift is then calculated by integrating these forces over space and time. This is certainly valid for gliding, but wing velocity and attitude are both continually changing in flapping flight. In the extreme case of hovering flight (Fig. 1), the wings travel a distance only a few times their

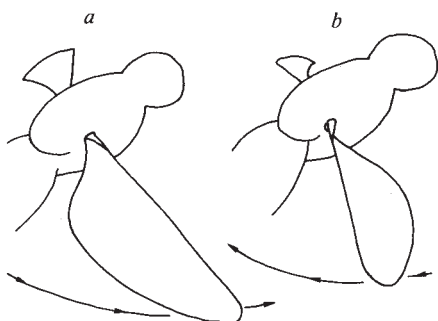


FIG. 1 The wingbeat of a hovering insect. The wings move back and forth, rotating by over 90° between the end of the downstroke (a) and the upstroke (b), and again at the end of the upstroke.

The authors have pointed the way towards a theoretical understanding of the collective effects and have shown that atom traps are important not only for the study of single-atom phenomena and atomic collisions, but also for the understanding of radiation hydrodynamics. □

Christopher Foot and Andrew Steane are in the Clarendon Laboratory, University of Oxford, Parks Road, Oxford OX1 3PU, UK.

own width during each beat before reversing their direction of travel and rotating by over 90°. Considering the problem of the Wagner effect, it is unlikely that this 'quasi-steady' assumption would be valid for hovering insects. Studies over the past twenty years of the aerodynamics of insects in free flight⁴⁻⁷ have usually concluded that the forces resulting from a conventional lift mechanism would not be adequate to support or propel the insect, and this has been verified by the results of Zanker and Gotz.

In addition, Zanker³ investigated the compensatory reactions of tethered flies in response to changes in visual stimuli (which would make the flies believe they were yawing, pitching or rolling) and changes in the speed of the air blown past them. Flies responded with changes in their wingbeat movements that in free-flight would have stabilized them, but conventional aerodynamic analysis of the altered wingbeat suggested that in some cases these responses would have made the flight more unstable. These insects must have been using different mechanisms both to produce lift and to manoeuvre.

The first unconventional lift mechanism to be discovered was the 'clap-and-fling' described by Torquell Weis-Fogh⁴. He noticed that in the flight of the tiny chalcid wasp, *Encarsia formosa*, the wings were always clapped together at the top of the upbeats of the wings before being rotated and so moved apart, leading-edge first (Fig. 2). He proposed that air rushing into the partial vacuum created as the wings separated would form attached circulations which would create large amounts of lift during the coming wing stroke. This effect has been experimentally verified, and in fact is widely used by flying animals. For instance, the noisy take-off by pigeons is caused by the dorsal clapping of their wings to produce the large amounts of lift they need.

Large values of lift also seem to be produced by animals after they rotate their wings at the start of a wingbeat, even when the wings are well separated. This occurs particularly when this rotation is

rapid and is delayed until after the wing has reversed its direction of motion. In hoverflies, for instance, the delay of wing rotation until after the start of the downstroke seems to enable them to produce large amounts of lift and allows them to hover when the wings are flapped through an angle of only about 50°, compared with 120° in most insects⁵. On the other hand, in higher flies such as *Drosophila*, the wings often bend downwards at the end of the downstroke at a line of weakness across the wing^{1-3,6,8,9} before flicking back up, leading-edge first. This causes fast, late rotation, which could enable them to generate large amounts of lift during the upstroke.

The mechanisms by which such isolated wing rotation can create circulation and lift are not fully understood, and until now this phenomenon has not been experimentally verified. Zanker and Gotz have achieved this by using flies tethered to a



FIG. 2 Wing movements generating unsteady lift during the clap-and-fling. The wings are clapped together (a) then rotated, leading edge first (b). Air rushes into the gap (broken arrows) and a circulation is set up around the wing for the coming stroke (c).

force transducer that could pick up a continuous record of the vertical forces produced during the wingbeat. They were able to show that in *Drosophila* peaks of lift production were produced not only after clap-and-fling at the top of the upstroke, but also after isolated wing rotation at the bottom of the downstroke.

Their results have two important implications. First, it is clear that to solve the problem of how insects control their flight will be extremely difficult; even if we discover exactly how the large numbers of direct flight muscles control the fine details of wing movement, we will not be able to solve this problem until we have a better understanding of unsteady aerodynamics. Second, studies of the aerodynamics of aerofoils in unsteady motion are urgently needed. Such investigation might not only clarify how animals fly, but would help us to improve our own aerodynamic designs; insects and birds are, after all, far more manoeuvrable than helicopters or aeroplanes. □

Roland Ennos is in the Department of Biology, University of York, York YO1 5DD, UK.

1. Zanker, J. M. *Phil. Trans. R. Soc. B* **327**, 1–18 (1990).
2. Zanker, J. M. & Götz, K. G. *Phil. Trans. R. Soc. B* **327**, 19–44 (1990).
3. Zanker, J. M. *Phil. Trans. R. Soc. B* **327**, 45–64 (1990).
4. Weis-Fogh, T. J. *exp. Biol.* **59**, 169–230 (1973).
5. Ellington, C. P. *Phil. Trans. R. Soc. B* **305**, 1–181 (1984).
6. Ennos, A. R. *J. exp. Biol.* **142**, 49–85 (1989).
7. Dudley, R. & Ellington, C. P. *J. exp. Biol.* **148**, 53 (1990).
8. Nachtigall, W. *J. comp. Physiol.* **133**, 351–355 (1979).
9. Wootton, R. J. *J. Zool.* **193**, 447–468 (1981).

Grass root destruction

Peter D. Moore

THE consumption of plant material by invertebrate herbivores can represent a considerable energetic loss for a plant. For example, in a study of the willow *Salix cinerea* in North Wales, 45 per cent of leaves were found to be damaged and roughly 13 per cent of the leaf area of the tree had been lost to invertebrate feeders¹; for smaller herbaceous plants subjected to heavy insect feeding pressure in grasslands, losses may prove even more serious. The long-term damage inflicted by insects on plant populations is assessed in two recent reports by Brown and Gange in *Oikos*² and in *Functional Ecology*³. Because selective consumption by insects results in severely reduced plant size, insect herbivory can increase the variability in individual shoot size in a population below ground; invertebrate grazers may further modify the composition of a plant community, and even influence its species diversity³.

Because of the complexity of plant-invertebrate interactions, many studies in this area have concentrated on single plant species and their response to a particular species of herbivorous invertebrate. Often such work is related to pest problems, or the possibilities of using plant-feeding insects for weed control⁴. But there are broader questions in community ecology that cannot be answered by such specific experiments. For example, what are the general effects of insect herbivory on a plant community and on the development of successions? If such questions were applied to mammalian grazers, the experimental technique that would spring to mind for their solution is the exclusion of the grazers from a sample plot and the observation of consequent changes in vegetation structure and composition in comparison with grazed areas. This experimental design is not so easily achieved for invertebrates, but at the Silwood Park field station of Imperial College, London, a series of long-term experiments has been initiated in which insecticides are sprayed on grassland plots from which the insects are to be excluded. These plots are selected to represent various stages in successional development, from one to 16 years.

Gange and Brown² describe the results from one experiment in which they investigated the influence of invertebrate herbivory on individual plant size. They found that plant size within eight species examined is generally greater in those plots where insecticide has been applied. There is also a smaller range in plant size in treated plots. So insect herbivory, by selectively damaging sensitive individuals, produces a skewed size frequency distribution among the plants. The influence of herbivory is thus operating in a manner similar to competition in that the less fit (that is, more palatable) individuals are reduced in size and thus placed at a disadvantage. Annual species respond rapidly, but in perennial species the effect is cumulative: loss of this year's photosynthetic tissue will affect next year's productivity. For this reason, size variability within a plant species becomes greater as succession proceeds.

Meanwhile, below the surface of the ground, root herbivory also takes its toll on plant tissues. This can be studied by similar methods to shoot herbivory, but using soil insecticides instead⁵. In this case, one of the most apparent effects on the plant community of removing soil insects is an increase in the number of plant species. Over a three-year succession from open ground, 83 species were recorded in treated plots compared with 70 in the controls. It would seem, therefore, that subterranean grazing is preventing the establishment of certain plant species. Brown and Gange point out that this constitutes an apparent contradiction of Grime's model⁶, in which above-ground grazing pressures increase diversity by reducing the dominance of robust plant species. But the early successional stage used in the Silwood Park experiment (up to three years) introduced factors of disturbance and a lack of stability into the

equation, that may make this comparison invalid.

What is evident, however, is that soil herbivory must be taken into account in studies of community development. This fact has been further underlined by findings in the Chihuahuan Desert in New Mexico⁶, where changes in the spatial distribution of the mesquite shrub (*Prosopis glandulosa*) in the past hundred years could be due to the intensity of attack by nematodes in the soil. In playa and arroyo habitats, in which the mesquite is becoming increasingly scarce, nematodes have been found down to depths of 12 metres, feeding upon even the deepest roots. Grassland and dune habitats are being invaded by mesquite, and here the depth to which nematodes penetrate is much smaller (less than 3 metres).

The study of what goes on below ground is proving an important development area in ecology, not only in the investigation of plant productivity and carbon sinks⁷, but also in the understanding of what controls the composition and successional development of plant communities. □

Peter D. Moore is in the Division of Biosphere Sciences, King's College, University of London, Campden Hill Road, London W8 7AH, UK.

1. Allende, M.C. *J. Ecol.* **77**, 1048–1058 (1989).
2. Gange, A.C. & Brown, V.K. *Oikos* **56**, 351–356 (1989).
3. Brown, V.K. & Gange, A.C. *Funct. Ecol.* **3**, 667–671 (1989).
4. Moran, V.C. & Hoffman, J.H. *J. appl. Ecol.* **26**, 967–977 (1989).
5. Grime, J.P. *Plant Strategies and Vegetation Processes* (Wiley, Chichester, 1979).
6. Freckman, D.W. & Virginia, R.A. *Ecology* **70**, 1665–1678 (1989).
7. Moore, P.D. *Nature* **348**, 858 (1989).

ATMOSPHERIC CHEMISTRY

Effects of CFC substitutes

Keith Shine

THE ratification in 1988 of the Montreal Protocol on protecting the ozone layer^{1,2} and the increasing pressure for its controls to be strengthened has naturally accelerated the search for potential substitutes to chlorofluorocarbons (CFCs). Although some companies are opting for non-halogenated molecules, much interest is being shown in a family of closely related molecules known generally as the hydrohalocarbons. No longer is there a presumption that such gases in the atmosphere are innocent until proven guilty and, in parallel with toxicity tests, their possible environmental effects are now being examined. The results of the first detailed study of the possible effects of the CFC substitutes on the ozone layer and greenhouse warming are reported in the two papers by Fisher *et al.* on pages 508 and 513 of this issue^{3,4}.

The so-called hard CFCs (CFC-11, -12, -113, -114 and -115) have the dubious priv-

ilege of playing important roles in both ozone depletion and greenhouse warming. The signing of the Montreal Protocol led to hopes that measures aimed at protecting the ozone layer would also lead to a reduction in the predicted warming. Wigley⁵ showed that adherence to the protocol could reduce the warming effects of the CFCs to between one-fifth and one-seventh of pre-Protocol projections. Wigley also considered a pessimistic picture in which the reductions in CFC-11 and CFC-12 emissions were made up for by emissions of one of the hydrochlorofluorocarbons, HCFC-22, a substitute already in widespread use; the reduction due to CFCs was four times greater than the increase due to HCFC-22. But more recent spectroscopic measurements have shown that the greenhouse strength of HCFC-22 was severely underestimated in earlier studies^{6,7}; an analysis similar to Wigley's, but

using the greenhouse strengths given by Fisher *et al.*⁴, reduces the gap to a factor of two.

In their two papers, Fisher *et al.* consider, in turn, the ozone depletion potential (ODP) and the halocarbon global-warming potential (HGWP) of the substitutes relative to CFC-11. Both measures require knowledge of each substitute's atmospheric lifetime: other things being equal, the shorter the lifetime, the lower the potential.

The vital difference between the CFCs and the hydrohalocarbon substitutes is that the substitutes retain at least one hydrogen atom. This makes them reactive in the lower atmosphere; consequently they have shorter lifetimes than the CFCs which are only destroyed in the stratosphere. Thus replacing the CFCs by the same emissions of substitutes leads to smaller atmospheric concentrations.

The ODP has a relatively long history and is enshrined in the Montreal Protocol. As a relative measure it is probably as good as a single number can ever be. The hydrofluorocarbons (HFCs) have a value of zero, as they contain no ozone-consuming chlorine at all; none of the HCFCs in the new analysis has an ODP exceeding 0.1 (ref. 3).

The HGWP is a much newer measure and it is important to distinguish between it and other measures of greenhouse strength. Gases can be compared by the greenhouse forcing they would cause molecule-for-molecule or kilogram-for-kilogram. Molecule-for-molecule, it is well known⁸ that the CFCs are about four orders of magnitude stronger than CO₂; by this measure, the substitutes are barely, if at all, weaker. Indeed, kilogram-for-kilogram, most are stronger than CFC-11 because of their relatively low molecular weights. An alternative measure is to compare the change in forcing for observed or projected changes in concentration; the large changes in the concentration of CO₂, some two to four orders of magnitude greater than for other greenhouse gases, puts it firmly at the top of the table, causing typically 50–60 per cent of the warming — this compares with the modelled contributions^{8,9} of CFCs to the warming in the 1980s of about 25 per cent.

The HGWP is rather different; it compares the effects of the emissions of a gas with the same emissions of CFC-11 and so is probably of more direct use to policy makers. Unlike the ODP, the spectroscopy of the carbon–fluorine bond means that fluorine is a very active component of the molecule's spectroscopic strength, so that the HFCs as well as the HCFCs must be considered. The results of Fisher *et al.*⁴ show that the HGWP of the potential substitutes ranges from 0.02 to 0.76 relative to CFC-11. Thus they are an improvement on CFC-11 and are mar-

kedly better than CFC-12 which, with a HGWP of around 3.0, is the main contributor of the hard CFCs to global warming.

However, the range of HGWPs given by Fisher *et al.*⁴ (their Table 5 see page 516) undoubtedly underplays the uncertainties involved, as the results have been normalized to a set of reference lifetimes. Most assessment models have been developed primarily for the study of the stratosphere and the representation of tropospheric chemistry is far cruder. A hint of the uncertainties involved can be seen from the lifetimes given in Table 2 (see page 510) of the ozone paper³; results from the two-dimensional models vary by a factor of two.

It is a simple matter to show that if the reference lifetimes are replaced with those calculated by the Atmospheric and Environmental Research Inc. (AER) two-dimensional model, the HGWP calculated by AER for HCFC-22 increases from 0.37 to 0.74 and that of HFC-125 increases from 0.65 to 1.27. It is evident from this that an increased understanding of tropospheric chemistry, and the role of the hydroxyl radical in particular, is of the highest priority.

The results of these two papers allow

QUANTUM ZENO EFFECT

Watching a laser hot-pot

Peter Knight

QUANTUM systems evolve through the establishment of coherent superpositions: an initial, unstable state develops into a superposition of undecayed and decay-product states whose relative weights are determined by the probability amplitudes for decay or survival. A measurement of survival projects the superposition back to the initial undecayed state; repeated, frequent measurement can inhibit or even prevent the decay. This is the quantum Zeno effect, or the quantum watched pot where the frequent intervention of an observer suppresses change or decay. A group of laser experimentalists at the National Institute of Standards and Technology in Boulder, Colorado, have performed an experiment using trapped ions in which such a suppression through measurement is observed (W. M. Itano *et al.* *Phys. Rev. A* **41**, 2295–2300; 1990).

The quantum Zeno effect has been of interest to theorists for some years as a striking example of the effect of measurement. B. Misra and E. C. G. Sudershan coined the name and identified the relevant timescales involved (*J. math. Phys.* **18**, 756; 1977). A critical time in a decay is the coherence time of the decay t_c , governed by the reciprocal of the range of energies accessible to the decay products. In most genuine quan-

um to see whether 'ozone friendliness' implies 'greenhouse friendliness'. The answer in my opinion is an emphatic no. It is difficult to know whether the volume of substitute production will ever approach the levels that had been anticipated for the hard CFCs; it is clear, however, that unrestrained emissions could lead to climatically significant concentrations during the next century^{5,7}. If the substitutes are to be used then prevention of unnecessary releases to the atmosphere will remain important. □

Keith Shine is in the Department of Meteorology, University of Reading, PO Box 239, Reading RG6 2AU, UK.

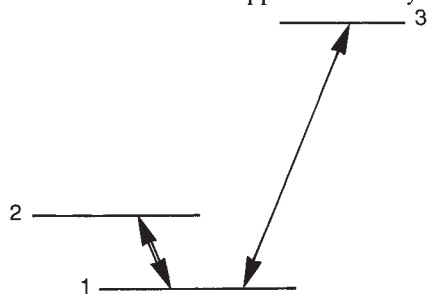
1. Montreal Protocol on Substances that Deplete the Ozone Layer (United Nations Environment Programme, Nairobi, 1987).
2. UK Stratospheric Ozone Review Group, *Stratospheric Ozone 1988* (HMSO, London, 1988).
3. Fisher, D.A. *et al.* *Nature* **344**, 508–512 (1990).
4. Fisher, D.A., Hales, C.H., Wang, W.-C., Ko, M.K.W. & Sze, N.D. *Nature* **344**, 513–516 (1990).
5. Wigley, T.M.L. *Nature* **335**, 333–335 (1988).
6. Varanasi, P. & Chudamani, S. *J. geophys. Res.* **93**, 1666–1668 (1988).
7. Shine, K.P. in *Ozone Depletion: Health and Environmental Consequences* (eds R. Russell Jones and T. Wigley) 71–83 (John Wiley, Chichester, 1989).
8. Ramanathan, V. *et al.* *Rev. Geophys.* **25**, 1441–1484 (1987).
9. Hansen, J., Lacis, A. & Prather, M. *J. geophys. Res.* **94**, 16417–16421 (1989).

tum decays (the spontaneous emission of a photon, nuclear α -particle decay, or β -decay in weak interactions for example) this range of energies is very broad and t_c is correspondingly short. If a quantum system is prepared in an unstable state, then after a reasonably long time t (but short enough for depletion to be unimportant), the probability of decay can be calculated from Schrödinger's equation.

If the elapsed time t is longer than t_c , this probability depends linearly on time and we talk of a time-independent rate γ for the decay: the decay probability is γt and the survival probability $(1-\gamma t)$. If, however, the elapsed time is less than t_c , we should not think of the evolution as an irreversible decay. It is, instead, a reversible evolution (not described by a rate) and behaves differently with time: the survival probability is $(1-at^2)$ where a is a collection of constants relevant to the transition. These entirely different evolution laws are crucial for the quantum Zeno effect.

Now imagine studying the quantum evolution for a total time T , but with repeated interrogations of the system by N measurements of the survival of the initial state at times T/N . The survival probability after one such measurement depends

on whether t is greater than t_c . If it is, the survival probability is $(1-\gamma(T/N))$, after N such measurements the probability of survival is $(1-\gamma(T/N))^N$ which for large N is approximately the exponential decay law, $\exp(-\gamma T)$, seen in every undergraduate radioactivity text. But the survival prospects dramatically improve if we interrogate the system repeatedly so that the time elapsed between each measurement is much shorter than the internal coherence time. In this case the survival probability after N such measurements is $(1-a(T/N)^2)^N$, which for large N becomes unity: the repeated measurements collapse the state to its initial state and suppress the decay.



Level scheme used in the quantum Zeno experiment. Be^+ ions are prepared in level 1 by optical pumping. A radio-frequency field is then applied for a time T to drive transitions from state 1 to a nearby hyperfine level 2. Measurements of the survival in state 1 are made with pulses of laser light at 313 nm exciting transitions from the initial state 1 to the strongly fluorescing high-lying state 3.

Decay suppression, therefore, depends on the internal coherence time of the system, but this is usually so short that no practical measurement can be made in a time $(T/N) \ll t_c$. This has ruled out, so far, any experimental realization of decay inhibition through measurement. What Itano and co-workers have done is to settle for a simpler proposal: to inhibit not a decay but a coherent transition induced between two essentially stable atomic states driven by a radio-frequency field. Such a system does not decay and its internal coherence time is essentially infinite. Evolution is always coherent and governed by the quadratic survival probability $(1-at^2)$ for reasonably short times. Repeated interruption and the inhibition of coherent evolution is much more straightforward (although it lacks the concept of an irreversible decay characteristic of the theoretical Zeno effect).

The experiment uses laser-cooled Be^+ ions in a Penning ion trap, and drives a radio-frequency transition (at 320.7 megahertz) between ground-state hyperfine levels. Optical pumping prepares the ions in the level marked as 1 in the figure. A pulse of resonant radiofrequency radiation is applied for a time T whose amplitude and duration — 256 milliseconds — is chosen to drive all of the ions out of state 1 into the state 2. The measurement of survival in state 1 is done with a laser pulse

at 313 nanometres wavelength which excites only transitions from the initial state 1 to a high-lying level 3 which strongly fluoresces. If at the time of application of the laser pulse, the ions survive in state 1, they can be driven into state 3 and the subsequent fluorescence observed. If, however, they have not survived, but have made the transition to state 2, they cannot be excited by the laser and no fluorescence is seen. The short laser pulse followed by photon counting of the fluorescence thus acts as a highly efficient measurement of the survival probability.

The number of measurements by the pulsed laser can be varied from one through to 64 within the 256 milliseconds of radio-frequency excitation. If only one is made, of course the survival probability is zero: the radio-frequency perturbation is chosen to excite all the probability out of state 1 into state 2. But if the system is interrogated by a short laser pulse half way through its coherent evolution as well the survival probability is seen to increase to one-half, in agreement with theory. As the number of measurements increase, the survival probability is seen to increase to nearly unity, indicating that the laser measurement repeatedly collapses the quantum evolution back to its initial state.

Whether the experiment tests the Zeno effect is a matter of taste: left to its own devices, the quantum system does not decay but coherently evolves. One could argue that inhibition of coherent evolution is rather less interesting and has been seen previously in laser excitation. For example, suppose the laser (the measurement apparatus) is left on continuously. Because the laser mixes into the initial state 1 some of the characteristics of the fluorescing level 3, the level 1 acquires an energy width Γ (which is the rate of stimulated transitions up to state 3 if the laser is not too strong). Then an elementary application of perturbation theory shows that the rate of radio-frequency transitions from a broadened state 1 to the state 2 is given by Ω^2/Γ where Ω is the Rabi frequency (the coherent evolution frequency) for the 1–2 radiofrequency transition. Increasing Γ reduces the radio-frequency transition rate. This is a well-known example of what is called power broadening. So the continuously observed coherent transition is well understood. But the repeatedly interrogated pulsed-excitation experiment tests a much more interesting feature of quantum dynamics: the destruction of coherent superpositions by measurement. This quantum watched pot is certainly prevented from boiling. $\square$

Peter Knight is in the Blackett Laboratory, Imperial College, Prince Consort Road, London SW7 2BZ, UK.

Play it again, Sam

LAST week Daedalus invented neuroseismology, a new way of reading the nerve traffic within the body. A nerve impulse produces a volume change moving along the nerve. This launches an ultrasonic disturbance into the tissues which, intercepted by an array of piezoelectric transducers on the skin, can be decoded to give the location, direction and velocity of the impulse. Proper electronic processing can even disentangle the very heavy impulse traffic in a multi-fibre nerve trunk, and can allocate every impulse to its originating fibre. Now Daedalus is taking the obvious next step. He is reversing the process, so as to induce pulses in the nerves.

The replay of a nerve recording back into the piezoelectric array would, of course, generate a zone of compression converging on the original nerve, and moving along it at the exact velocity and in the same direction as the original impulse. A nerve can be made to fire by squeezing it: this is what causes the 'pins and needles' sensation in awkwardly trapped or pressured limbs. So the replayed neuroseismogram should launch in the nerve a copy of the original impulse. An array that could spatially resolve the signals of the many fibres of a nerve trunk could also, in reverse, address them individually with the same resolution. The most complex original nerve traffic could be played back in full detail.

The obvious application of the new technology is the recording of human sensation. Why bother with videorecorders or tape machines when the nerve traffic of your own eyes and ears can be captured and replayed exactly? In principle, all the sensory delights of youth could be stored up and savoured into old age, a true *recherche du temps perdu*. In practice, however, the body grows and changes, the piezoelectric array could never be re-sited exactly, and the replay would hit the wrong nerves, giving a grotesque distortion of the original experience. Only an immediate replay could truly succeed.

The most immediate replay of all would give a neurological amplifier. A piezo-helmet that read the nerve traffic of the cranial nerves and spinal cord, and played it all back one or more times with a slight delay, would follow up every nerve impulse with one or more 'echoes'. Since the nervous system works by pulse-integration, this in effect would turn up its 'gain'. The ears would become sharper, the eyes more sensitive, taste and smell more vivid, the muscles more responsive. The user's whole world would be brighter and more dynamic. He could see in deep gloom, or lift astonishing weights, by increasing the relevant gain still further: until system noise, in the form of random sensory flicker or muscle tremor, stepped in to limit his powers.

David Jones

InsP₃ receptor turnaround

SIR—Two recent papers, one by us¹ and one by Furuichi *et al.*², report the similarity in primary structure between the 1,4,5-triphosphate (InsP₃) receptor and the ryanodine receptor³, both gated Ca²⁺ channels. Although the amino-acid sequences reported in the two papers fully agree, there is disagreement about the topology of the InsP₃ receptor in the membrane, and the subcellular localization of the protein in cerebellar Purkinje cells.

Although Furuichi *et al.* do not define the number of transmembrane spans of the molecule, they conclude that the C terminus is not located in the cytoplasmic compartment. This contrasts with the cytoplasmic localization of the C terminus of the ryanodine receptor. They also conclude that the InsP₃ receptor protein is localized not only in the endoplasmic reticulum (ER), but also on the plasmalemma including postsynaptic densities. Here we point out why the results of our study argue strongly against these conclusions.

The portions of the ryanodine receptor and of the InsP₃ receptor close to the C termini show primary structure similarity and are those thought to be involved in Ca²⁺ translocation. Therefore, as pointed out in the News and Views article that discussed both papers⁴, an opposite localization with respect to the membrane of the C termini of the two proteins is unexpected. Although not emphasized in our original report, our immunocytochemical experiments¹ unambiguously indicate that the C terminus of the InsP₃ receptor, like the C terminus of the ryanodine receptor, is localized in the cytoplasm. Our results show that antibodies raised against a synthetic peptide corresponding to the 19 C-terminus residues of the molecule bind to the cytoplasmic surface of intact (nonpermeabilized) ER tubules and cisternae.

If the InsP₃ receptor is concentrated both in the ER and in the plasmalemma, it would be the first example of a transmembrane protein with such a subcellular distribution. The prevailing concept in cell biology is that transmembrane proteins after synthesis are either retained in the ER, or exported to other membranes of the vacuolar apparatus or to the plasmalemma⁵. Our immunocytochemical experiments do not provide any evidence for the presence of the InsP₃ receptor (at

least the InsP₃ receptor in question) at the cell surface including postsynaptic densities. In our immunogold procedure, both the outer and the inner surface of the plasmalemma were accessible to antibody binding, because of mechanical cell dissociation and elution of cytosolic proteins. No immunocytochemical data were shown by Furuichi *et al.* They quote a previous study performed by an immunoperoxidase technique⁶. Because nonspecific labelling of membranes next to immunoreactive sites is a well-known artefact of the immunoperoxidase technique (see ref. 7), the immunoperoxidase results quoted by Furuichi *et al.* are likely to be explained by the presence of InsP₃ receptor-containing ER membranes next to the plasmalemma. Incidentally, a previous immunoperoxidase study on the localization of the InsP₃ receptor⁸ also did

not reveal any InsP₃ immunoreactivity on the plasmalemma.

To conclude, there is as yet no evidence for assuming that two structurally and functionally similar protein domains have different topologies in the membrane, and rejecting the 'dogma' that a transmembrane protein is destined to be either retained in, or exported from, the ER.

PIETRO DE CAMILLI
KOJI TAKEI

Department of Cell Biology,
and Section of Molecular Neurobiology,
Yale University School of Medicine,
PO Box 3333,
New Haven,
Connecticut 06510, USA

GREGORY A. MIGNERY
THOMAS C. SÜDHOF

Howard Hughes Medical Institute and
Department of Molecular Genetics,
University of Texas Southwestern
Medical Center,
Dallas, Texas 75235, USA

Mating calls

SIR—Ryan *et al.*¹ report the basilar papilla tuning of female frogs of the species *Physalaemus coloradum* to be no different from that of the closely related *P. pustulosus* (Tungara frog), and take that as evidence for a "sensory exploitation" hypothesis for an evolutionary origin of a low frequency male chuck emitted by *P. pustulosus*. The hypothesis is that the tuning of the female ear evolved before the chuck, which has been naturally selected in *P. pustulosus* secondary to the physical characteristics of the female ear. Lack of a chuck in *P. coloradum* seemed to be evidence against alternative hypotheses that assume the male trait evolved in coordination with the female trait.

The argument depends on the assumption that *P. pustulosus* derived the chucking capacity after species divergence. Alternatively, the female ear tuning and the male chuck could have evolved together in an ancestral species and the chuck would have been lost by differential predation pressure on males of *P. coloradum*. Ryan demonstrated that there was heavy predation pressure on the Tungara frog from frog-eating bats, which preferentially key on the low-frequency complex chucks rather than on the simpler high-frequency sounds these frogs also make². Selection pressure would be greater on a male vocalizing apparatus than on the silent female ear. Sensitivity to low frequencies is therefore more likely to have become a conserved attribute, with no apparent present use, than emission of the same low frequencies.

This explanation of the results has the advantage of providing a mechanism for the evolution of the female basilar papilla in an ancestral species. Support for the argument comes from the evolutionary

history of the Tungara frog, which is hard to study. The divergence apparently began in the late Tertiary, after the formation of the Andes. The two species gained their separate characteristics on either side of the continental divide. No fossil remains of a primordial species are described, much less its vocal apparatus³.

But the hypothesis that the loss of the trait by predation needs to be clearly disproven before the sensory exploitation hypothesis gains plausibility.

RUSSELL GARDNER

Department of Psychiatry,
University of Texas Medical Branch,
1.200 Graves Building,
Galveston,
Texas 77550,
USA

1. Ryan, M.J. *et al.* *Nature* **343**, 66–67 (1990).
2. Ryan, M.J. *The Tungara Frog: A Study in Sexual Selection and Communication* 83 (Chicago: University Press, 1985).
3. Cannatella, D.C. & Duellman, W.E. *Copeia* 1984: 902–321, 1984.

SIR—In the interesting article by Ryan *et al.*¹, the conclusion that there has been no evolution of female preference follows from the assumption that tuning of the peripheral auditory system accurately predicts female mate preference. A simple way of testing this would be to expose female *Physalaemus coloradum* to calls of both species in a classic two-way loudspeaker choice experiment. We would expect *P. coloradum* females to prefer the call of *P. pustulosus* males over conspecifics. Alternatively, both these species may share the same basilar papilla tuning, but differ in female response to auditory stimulation. In that case, female preference would differ in the two species (say, if *P. coloradum* females ignored chucks), and the relevant selective pres-

1. Mignery, G.A., Südhof, T.C., Takei, K. & De Camilli, P. *Nature* **342**, 192–195 (1989).
2. Furuichi, T. *et al.* *Nature* **342**, 32–38 (1989).
3. Takeshima, H. *et al.* *Nature* **339**, 439–445 (1989).
4. Gill, D.L. *Nature* **342**, 16–18 (1989).
5. Warren, G. *Nature* **327**, 17–18 (1988).
6. Maeda, N. *et al.* *Dev. Biol.* **133**, 67–76 (1989).
7. De Camilli, P., Harris, S.M., Huttner, W.B. & Greengard, P. *J. Cell Biol.* **96**, 1355–1373 (1983).
8. Ross *et al.* *Nature* **339**, 468–470 (1989).

tures would remain to be elucidated.

Such an experiment may confirm Ryan *et al.*'s hypothesis that female preference has not evolved at all, but sensory exploitation alone cannot explain the evolution of either the male call or the maintenance of female preference. The male chuck is notable because its mean frequency is higher than the peak female receptivity; a signal evolved solely through sensory exploitation should coincide with peak receptivity, not fall short of it. It seems likely to us that the shortfall in the male chuck is the result of counter-selection against lower frequency calling (perhaps deep calls are costly), implying that processes other than sensory exploitation are important. Alternatively, but less plausibly, there may be some genetic constraint on the evolution of deeper chucks.

For these reasons, we think it unlikely that sensory exploitation can offer a genuinely alternative explanation to that of the evolution of female preference through sexual selection. In a sense, all signals must evolve along routes that exploit sensory biases in the signal receiver. The crucial question is not whether sensory exploitation has been involved, but why females have allowed themselves to be exploited. Ryan *et al.* suggest this is because mate preference is fixed, presumably by genetic constraints, yet there is plenty of evidence that preferences can evolve in anurans^{2,3}. We believe it is far more likely that females benefit through their mate preference because properties of the male chuck (such as frequency) contain useful information about male quality. If this were not the case — if, for example, sensory bias led females to prefer infertile males — then selection would act to extinguish such a preference.

Indeed, Ryan *et al.* themselves report one direct advantage of preference: females have more fertilized eggs when they mate with larger males and larger males have lower frequency chucks. There may also be other heritable benefits, such as those proposed in the Darwin-Fisher (attractive sons) or the 'good genes' (high offspring survival) views of sexual selection. Neither of these hypotheses can be ruled out by the reported data. Sensory exploitation is not a rival of these hypotheses. The evolution of signals and their reception will occur not only through sensory exploitation, but also through selection on the signal receiver and signal producer.

ANDREW POMIANKOWSKI
TIM GUILFORD

Department of Zoology,
University of Oxford,
Oxford OX1 3PS, UK

1. Ryan, M.J. *et al.* *Nature* **343**, 66–67 (1990).
2. Hoy, R.H., Hahn, J. & Paul, R.C. *Science* **195**, 82–84 (1977).
3. Ryan, M.J. and Wilczynski, W. *Science* **240**, 1786–1788 (1989).

Superficial microwave heating

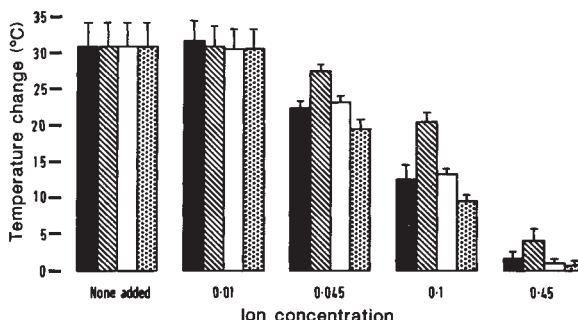
SIR—The domestic market for microwave ovens has grown rapidly in Britain since

meals investigated were 0.024–0.15 M sodium (mean 0.102 M); 0.012–0.12 M potassium (mean 0.036 M). These concentrations are clearly in the range that would be affected.

The poor penetration of microwaves into the test food with high ionic concentrations may result from the induction of electrical/ionic flow in the surface of the food. This would also explain why commercial food heated in microwaves commonly boils on the surface but is cool on the inside.

STEPHEN F. DEALLER
RICHARD W. LACEY

Department of Medical Microbiology,
Leeds University,
Leeds LS2 9JT,
UK



The temperature change induced at the core of 200 g mashed potato by a 650-W microwave oven in 1 minute versus the concentration of ionic compounds present in the food. The ranges indicate one standard deviation. Filled columns, NaCl; striped, monosodium glutamate; open, NH₄Cl; stippled, KCl.

the early 1980s, helped by advertising slogans such as "heats from the inside out". But there were no scientific data to support these claims. The recent increase in salmonellosis and listeriosis has suggested that the bacteria causing these diseases, if present in food¹⁻³, are not being killed by reheating or cooking. We have measured the change in temperature of food as a measure of energy transfer to it from microwaves and have found that such temperatures are not adequate reliably to kill bacteria present⁴. We wondered whether the apparent contradiction could be caused by high ionic concentrations in the food, allowing the induction of ionic currents by the microwaves and causing energy to be released, preventing the microwaves from penetrating the food to heat the core.

We made mashed potato with no added salts, measured its ionic content and cooled it to 8 °C. We added dissolved chemical salts (NaCl, KCl, NH₄Cl and monosodium glutamate) to some samples. Each sample was put onto the centre of the rotating turntable of a Matsui 170 TC 650-W, 1250-MHz domestic microwave oven and heated for 1 min. The core temperature was measured directly after heating. We repeated the experiments using 300 g mashed potato in a cook-chill microwave oven, and we also investigated some ready-prepared meals.

The core temperature change of the mashed potato in the beaker decreases with increasing concentration of the added salts (see figure). In the microwave oven dish, the temperature change of the food with added salts was about 62 per cent of that when no salts were added. The concentrations of sodium and potassium in the 10 commercial ready-processed

Irradiated cells

SIR—The association of childhood leukaemias and fathers' occupational exposure to radiation reported by Gardner *et al.*¹ raises the possibility that if workers were contaminated with unidentified radionuclides, which may be enriched in the urogenital organs or the semen, these would not be detected by external dosimeters.

An objective of the late Radiological Protection Service MRC Stable Element Unit was to determine the abundances of elements in human tissues, organs and their routes of entry in relation to those for radioactive analogues². For the radionuclides of the heavy alpha-emitting radionuclides, which are often associated with small volumes or surfaces, suitable stable-element analogues are the rare-earth elements and possibly barium-sulphur pathways. Rare-earth elements have been detected in bone, liver, kidney, lymph nodes and testis, but not in ovaries, using autopsy samples following accidental deaths. Wester³, using neutron-activation analysis, also found cerium and lanthanum in RNA and the sacrotubular fraction of beef heart tissue.

If a single-hit hypothesis for the induction of a cancer by alpha-emitters is accepted, then it seems likely that damage to spermatogonia or their stem cells is possible. Today the concept of multiple hits is not considered likely, even in the instance of irradiation of cells by 'hot' particles for which high concentrations of radionuclides are associated with small volumes. Nevertheless, the subcellular distribution, or surface enrichment, of

1. Lancet **231**, 720–722 (1988).
2. Gellin, B.G. & Broom, C.V. *J. med. Ass. Am.* **261**, 1313–1320 (1989).
3. Rampling, A. *et al.* *Lancet* **334**, 436–438 (1989).
4. Sheeran, M.R.M. *et al.* *J. hosp. Infec.* **14**, 84–86 (1989).

alpha-emitting and other radionuclides following environmental exposure is poorly known, with the exception of hot particles in lung tissue arising from the decay products of radon-222.

Analysis of gross tissues or organs is unlikely to be useful if a particular radionuclide is associated with a small area or volume of cells. Possible biochemical pathways of interest are the trapping of rare earths, such as cerium by phosphate ions which are released by phosphatases and an association with the intracellular protein ferritin.

So far there is no indication that localization of heavy radionuclides in human testes occurs; concentrations of other conservative radionuclides present in the systemic circulation are unlikely to be of concern in relation to uniform distributed dose received by the testis. However, an inert element, such as thorium, can be detected in human blood and urine and is enriched in bone for occupational and non-occupational exposure¹.

E.I. HAMILTON

Phoenix Research Laboratory,
Pengele, Dunterton,
Milton Abbot,
Tavistock,
Devon PL19 0QJ, UK

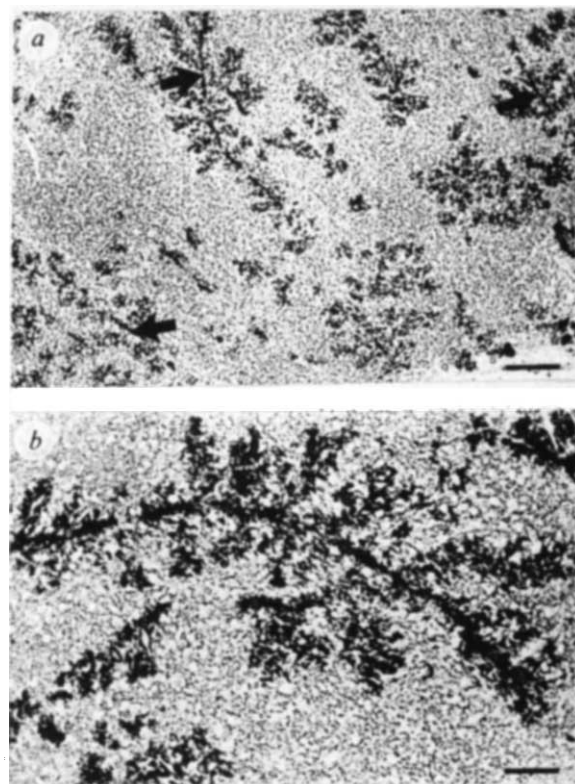
1. Gardner, M.J. *et al.* *Br. med. J.* **300**, 423–429 (1990).
2. E.I. Hamilton, *The Chemical Elements and Man* (Thomas, Springfield, Illinois, 1979).
3. Wester, P.O. *Sci. Total Environ.* **1**, 100–101 (1972).
4. Clifton, R.J., Farrow, M. & Hamilton, E.I. *Ann. occup. Hyg.* **14**, 303–308 (1971).

Cortical β -amyloid

SIR—The presence of amyloid β -protein, the essential component of the amyloid substance found in the brains of patients with Alzheimer's disease¹, has been demonstrated by a variety of immunohistochemical techniques². More recently, amyloid β -protein has been found in tissues other than brain (for example, skin, subcutaneous tissue and intestine), in conformity with the hypothesis that there is a circulating form of the β -protein³.

The improved sensitivity of the immunohistochemical techniques that results from pretreatment of nervous tissue with formic acid, has revealed amyloid deposits that have infiltrated the grey matter of the cortex of patients with Alzheimer's disease⁴. We have achieved a further improvement of sensitivity in the detection of amyloid deposits by a technique involving pretreatment with periodic acid, the effect of which is to remove

Amyloid deposits in the white matter of the occipital cortex of a patient with Alzheimer's disease who died at 75 years are revealed by immunolabelling with antibodies against synthetic peptides. Amyloid deposits associated with capillaries are denoted by arrows. Scale bars: a, 100 μ m; b, 50 μ m. Tissue was fixed in Carnoy's mixture and paraffin sections were treated with periodic acid before immunolabelling⁵. Similar observations were made with two other patients who had died at 77 years, but no amyloid deposit was detected in the cerebral white matter of three aged controls who died at 80 years.



saccharides, which are known to interact strongly with all amyloid substances⁵.

Using this technique, we have been able to demonstrate amyloid substance in the immediate vicinity of capillaries in the cortical white matter of patients with Alzheimer's disease (see figure). To our knowledge, this is the first report of amyloid β -protein in cortical white matter. The observation agrees with the

hypothesis that amyloid β -protein is vascular in origin.

N. BEHROUZ
A. DEFOSSEZ
A. DELACOURTE
M. MAZZUCA

Laboratoire d'Histologie
Unité Inserm 156 ADERMA,
Place de Verdun, 59045
Lille Cedex, France

Collagenous proteins multiply

SIR—The collagen stalk motif of three amino-acid residues, Gly-X-Y, has been found not only in extracellular matrix collagens but also in other eukaryotic secretory proteins such as complement C1q, pulmonary surfactant apoprotein, asymmetric acetylcholine esterase and serum mannose-binding protein. Now it has also been demonstrated that the motif is used in a non-secretory protein — the macrophage scavenger receptor^{1–3}, a membrane protein with important implications in atherosclerosis.

We are concentrating on studies of bacterial viruses with a membrane⁴. One of these, the *Escherichia coli* bacteriophage PRD1, has an external icosahedral protein coat, composed of major and minor capsomers, surrounding the viral membrane. We have very recently discovered that the minor capsomer protein (34.3 K) forms an asymmetric homotrimer where amino-acid sequence, deduced from the DNA sequence of the minor capsid protein gene, reveals a carboxy-terminal domain of 199 amino acids including all the three cysteine residues of

the protein, and an amino-terminal domain 123 amino acids long.

These two domains are separated by a short collagen-like region with 6 continuous Gly-X-Y triplets. Four out of the 6 Y amino acids are proline or lysine. In addition, microbial collagenase cleaves the protein into the carboxy- and amino-terminal domains⁵.

We believe that this trimeric minor capsomer of PRD1 has a structural role and that the short collagen-like motif assures the multimerization needed to satisfy the symmetry requirements. Our finding suggests that this peptide motif is evolutionarily old, and that it may be found in a number of trimeric proteins.

D.H. BAMFORD
J.K.H. BAMFORD

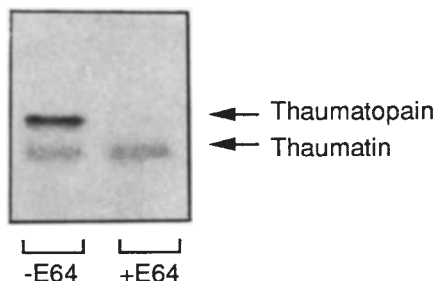
Department of Genetics,
University of Helsinki,
Helsinki, Finland

1. Glenner, G.G. & Wong, C.W. *Biochem. biophys. Res. Commun.* **120**, 8885–8890 (1984).
2. Ikeda, S.-I., Allsop, D. & Glenner, G.G. *Lab. Invest.* **60**, 113–122 (1989).
3. Joachim, C.L., Mori, H. & Selkoe, D.J. *Nature* **341**, 226–230 (1989).
4. Kitamoto, T., Ogomori, K., Tateishi, J. & Prusiner, S.B. *Lab. Invest.* **57**, 230–236 (1987).
5. Behrouz, N., Defossez, A., Delacourte, A., Hublaur, P. & Mazzuca, M. *Lab. Invest.* **61**, 576–583 (1989).

1. Kodama, T. *et al.* *Nature* **343**, 531–535 (1990).
2. Rohrer, L. *et al.* *Nature* **343**, 570–572 (1990).
3. Brown, M.S. & Goldstein, J.L. *Nature* **343**, 508 (1990).
4. Mindich, L. & Bamford, D.H. in *The Bacteriophages 2* (ed. Calendar, R.), 475–519 (Plenum, New York, 1988).
5. Bamford, J.K.H. & Bamford, D.H. *Virology* (in the press).

Thaumatococcus not proteolytic

SIR—Skern *et al.*¹ have recently called to attention a short region of homology between the sweet protein thaumatin, trypsin and a group of picornaviral proteases, proposing that this similarity may explain the apparent proteolytic



A crude aqueous extract of *Thaumatococcus daniellii* arils was activated with 30mM 2-mercaptoethanol, one half of the sample was treated with 100 μ M E64 (+E64) such that no residual activity towards ZFRNec was measurable. Both samples were then treated with 30 μ M [¹⁴C] iodoacetate for 4 minutes, at which point unlabelled iodoacetate was added. The preparations were then separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and subjected to autoradiography. Despite a 100:1 ratio of thaumatococcus:thaumatopain in the crude extract, thaumatopain is preferentially labelled to a high level, provided the thaumatopain preparation was not previously inhibited by E64 (–E64). Incorporation of label into thaumatococcus is not E64-sensitive.

activity of thaumatococcus, and conjecturing that thaumatococcus has cysteine protease activity². They suggest that treatment of thaumatococcus with a reducing agent such as dithiothreitol breaks an exposed disulphide bond and generates a nucleophilic cysteine residue.

Such a cysteine protease activity would indeed be unusual; thaumatococcus contains no histidine residues³ and thus lacks the normal catalytic triad that facilitates the activation of the nucleophilic cysteine residue⁴. But we have obtained evidence that the proteolytic activity of thaumatococcus is an artefact⁵.

During our earlier work on the structure/sensory properties of thaumatococcus, we observed that purified preparations (Talin, a gift from E. Rathbone) have significant amidolytic and proteolytic activity (towards Z-Phe-Arg-7-amido-4-methylcoumarylamide and radioiodinated insulin B chain, respectively). Both activities required prior activation with

reducing agents, and were inhibitable with classical cysteine-reactive reagents such as iodoacetate and the highly specific cysteine protease inhibitor E64. We used E64 to show that the amidolytic activity was completely suppressed after addition of 10 pmol E64 to a preparation containing 1 nmol thaumatococcus. We conclude that the cysteine protease activity in thaumatococcus preparations can be attributed to trace amounts of a contaminating protease, and that thaumatococcus itself is not a protease⁵.

We have since isolated the cysteine protease, referred to as thaumatopain, from arils of *Thaumatococcus daniellii* (the source of thaumatococcus); it has a relative molecular mass of 30,000 and shares many properties with classical cysteine proteases such as papain (M. C. *et al.* manuscript submitted). In crude aril extracts, thaumatopain is the source of the most nucleophilic cysteine residue. It is more basic than thaumatocosses, which may explain why the more basic thaumatocosses preparations (isolated by low resolution ion exchange chromatography) were

reported to possess greater proteolytic activity². Different batches of thaumatococcus also differ in their ability to undergo autolysis (Dowdell and R. B. unpublished data).

We have searched the PIR and Swiss-Prot databases for the 'active-site' sequences GDSGG and GDCGG and found several matches to proteins not considered to be proteinases. We agree with Skern *et al.*¹ that apparent similarities may mislead and must be interpreted in the light of other experimental data. Such data suggest that thaumatococcus itself is not proteolytically active.

R. BEYNON

Proteolysis Group,
Department of Biochemistry,
University of Liverpool,
PO Box 147,
Liverpool L69 3BX, UK

M. CUSACK

Department of Geology and
Applied Geology,
University of Glasgow,
Glasgow G12 8QQ, UK

Immoral wasps?

SIR—The caption to the photograph of *Polistes* on the cover of your issue of 16 November last year says that wasps can be 'altruist'. That some actions by a wasp (or an ape, or a man) can be objectively described as increasing the fitness of another at the expense of the actor's own is not the point at issue. Ever since the classic paper of W. D. Hamilton^{1,2}, such acts have been called 'altruistic behaviour', and the definition has become standard in evolutionary ethology.

Nevertheless, when teaching these matters, I have become increasingly uncomfortable about the use of the word 'altruism' in this context. If we agree that it is an important endeavour to afford an evolutionary explanation of human behaviour, we should acknowledge that the eventual synthesis will have to reconcile the points of view of biologists and of social scientists. In my experience, reconciliation with the usage of social scientists will stumble on this word even when it is carefully defined, because we "use the word in a restricted . . . sense, only superficially related to common usage"³.

There is no inconvenience in using the same word for two clearly distinct meanings in fields that are also clearly distinct. Nobody would ever suggest that the charms of Madonna, say, are directly related to those of her constituent quarks. But extending the concepts of animal ethology to human behaviour implies that we are ready to use the same unambiguous vocabulary throughout the field.

Ethology aims at describing and understanding behaviour in terms as objective as possible and, in any case, without connotations of moral value. But

when anybody, even a sociobiologist, calls a human action "altruistic", he clearly refers to the moral value of that action. His statement implies reference to a cultural scale of moral values as well as a recognition of the responsibility of the actor. Neither a wasp nor an ape, or even an insane man, can be regarded as morally responsible and, when it comes to human behaviour, explaining is not the same as judging.

It follows that, if we are to include the study of human behaviour within comparative ethology, we have to respect the rules of priority and of clarity by which we abide in biology. Priority in the use of 'altruism' in its ethical sense cannot be disputed. I therefore propose that in ethology and evolutionary biology we coin a new word, as free as possible of ethical connotations, to designate those behaviours that, on a strictly descriptive level, resemble altruism.

A good candidate would be "euxeny" (euxenic), from the greek ευξενικός, meaning "favourable to strangers, hospitable". To my knowledge, the word is used neither in biology nor the social sciences. I appreciate that changing a habit of 25 years would not be easy, but if in doing so we avoid some pointless disputes about sociobiology, the effort might be worthwhile.

RAYMOND RASMONT

Laboratory of Animal Biology,
University of Brussels,
50 Ave. F. Roosevelt,
1050 Brussels,
Belgium

1. Skern, T., Zorn, M., Blaas, D., Kuechler, E. & Sommergruber, W. *Nature* **344**, 26 (1990).
2. Van der Wel, H. & Bel, W.J. *Eur. J. Biochem.* **104**, 413–418 (1980).
3. Lee, J.-H. *et al. Biochemistry* **27**, 5101–5107 (1988).
4. Dunn, B.M. in *Proteolytic Enzymes — A Practical Approach* (eds Beynon, R.J. & Bond, J.S.) 57–81 (Oxford Univ. Press, 1989).
5. Cusack, M., Beynon, R.J. & Rodgers, P.B. *Biochem. Soc. Trans.* **15**, 880 (1987).

1. Hamilton, W.D. *Am. Nat.* **97**, 354–356 (1963).
2. Hamilton, W.D. *J. theor. Biol.* **7**, 1–52 (1964).
3. Dawkins, R. *The Extended Phenotype* (Oxford Univ. Press, 1982).

Working towards one world

Norman Myers

International Resource Management. Edited by S. Anderson and W. Ostreng. *Belhaven*: 1989. Pp. 301. £30, \$40.

Environmental Data Report, 2nd edn. By the United Nations Environment Programme. *Blackwell*: 1989. Pp. 547. £45, \$39.95.

Global Issues in the United Nations' Framework. Edited by Paul Taylor and A. J. R. Groom. *Macmillan*: 1989. Pp. 371. £29.50, \$45.

In Fairness to Future Generations: International Law, Common Patrimony, and Inter-generational Equity. By Edith Brown Weiss. *Transnational Publishers*: 1988. Pp. 385. \$59.50.

AFTER postulating that the environmental prospect presents a threat second only to a nuclear exchange, the authors of the Brundtland commission report reflected "Our earth is one, our world is not." At a time when we are undermining the very life-support basis of the planet — a situation that demands an unprecedented degree of collaboration on the part of all humankind — we still run our planetary affairs through the medium of the nation-state system. True, we have sundry international agencies to articulate the collective good. But they tend to be intergovernmental committees, with all the competitive, as opposed to cooperative, spirit that has long characterized nation states. The required shift in international relations will represent as great an advance in the nation-state system as any since the emergence of nation states 400 years ago.

The editors of *International Resource Management* consider various institutional regimes that have attempted to reconcile the needs of individual nations with those of the community of nations. Fourteen experts draw on their experience to illuminate the challenges, looking at such common resources as Antarctica, the atmosphere, the ozone layer, the North Sea, marine fisheries and the great whales. The picture is decidedly mixed: a few successes, several semi-successes and many failures. For those seeking an overview of our halting efforts at international management of common property, this is a sound assessment, albeit without much in the way of a synthesizing appraisal of commonality factors at issue. The book would have been much better had the editors provided a concluding analysis of where we have got to and what lessons have been learned.

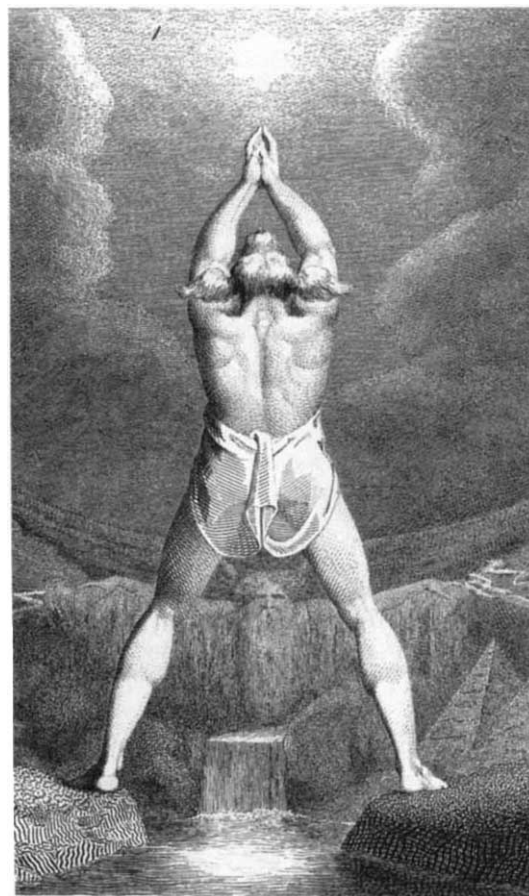
A pervasive theme of the book is the role of the scientist in advising policy makers, especially in those fields, becoming more frequent, where we can no longer afford the luxury of a conventional level of scientific evidence before taking incisive action. The case of the ozone layer is instructive: if policy makers had only felt they could respond in the early 1980s, when the problem was first adumbrated, we could have saved damage to the

atmospheric layer that will impose injury to humans and biotas for decades to come. Yet a safety-first approach was deemed unjustified, on grounds both of insufficient scientific "proof" and incapacity of international institutions to take anticipatory and preventive measures. Governments are essentially reactive bodies, waiting for the morning mail to deliver its new problems and then bestirring themselves with curative responses — even though the cure may well be expensive and less than effective. Fortunately, we can take heart from the recent initiatives to limit chlorofluorocarbons, amounting as they do to a first international effort to counter a global threat that will not manifest itself in full scope for a good time to come.

The *Environmental Data Report* contains an abundance of data on our environmental predicament, and thus reveals our growing capacity to build a science base on which policy makers can rest their decisions. The United Nations Environment Programme (UNEP) publishes periodic surveys of pollution, climate change, natural resources, population and settlements, energy, wastes and natural disasters among other environmental phenomena. As a compendium of information, mostly of mid-1980s vintage, this book should serve as a fine reference source for those who want a quick insight into global patterns and trends of environmental decline. But it is curiously patchy in its coverage, with next to nothing on deforestation, soil erosion, desertification or mass extinction of species. Next time, UNEP? We can't wait to know the answer!

UNEP emerged as the principal agency to be set up in the wake of a series of United Nations mega-conferences during the 1970s. The United Nations system was established to tackle postwar problems. It turned out to be poorly adapted to confront the growing problems of the developing countries and international relations, so new institutional initiatives became necessary. Hence the world conferences on environment, population, food, women's rights, energy and international economic reform, among others. Some of these proved productive in terms of concrete responses, others far less so.

Global Issues discusses the capacity of the United Nations to grapple with problems that are intrinsically global in nature and extent. The editors present a systematic analysis of the potential and shortcomings of the United Nations in the global arena — without much good news about its operational capacity to devise responses of appropriate scope and scale. Not surprisingly, the nation-state system remains the chief stumbling block. Yet even if we were to abolish nation states,



Life-source — William Blake's *Fertilization of Egypt* (1795), in which Sirius, the Dog Star, is depicted as the sign of the summer rise and flooding of the Nile, vital to the fertility of the flood plain. The picture is reproduced from *The Teaching of Astronomy* edited by J. M. Pasachoff and J. R. Percy, published by Cambridge University Press, price £35, \$54.50.

we would have to invent something similar, as we need multiple "management packages" to implement global policies at less than global level.

The last book in this collection is the most substantive, perhaps because it is the only one by a single author. Weiss points out that the international legal order has been preoccupied with measures to take care of contemporary problems, yet our environmental challenges increasingly reach far into the future. Depletion of the ozone layer and global warming will entrain impoverishing repercussions for several generations to come, whereas mass extinction of species will leave a depauperate biosphere for perhaps a million years. How can the international community encompass the interests of future humankind on such a broad front?

Weiss examines the complexities of interdependence in this new temporal dimension, reviewing our understanding of rights, obligations, liability and "justice" as applied to nuclear waste, biological resources, water, soils and climate. A pioneering endeavour in a field of ultra-urgency, Weiss presents a first-class statement of juridical intricacies, together with strategies for implementation such as codification, finance, management and incentives. From start to finish the book is informative and stimulating — hurrah, too, for the jargon-free style! The Brundtland commission would have found it an illuminating account of how to start to make our world one. □

Norman Myers is a consultant in environment and development at Upper Meadow, Old Road, Oxford OX3 8SZ, UK.

Times of change

John Bancroft

Menopause: Evaluation, Treatment and Health Concerns. Edited by Charles B. Hammond, Florence P. Haseltine and Isaac Schiff. *Liss: 1989. Pp. 339. \$96, £76.*

THE medical management of the menopause is an issue of growing interest and importance. As the evidence accumulates the complexity increases. From what was traditionally regarded as "the change", a normal developmental process sometimes with associated problems, we have now a situation where the long-term health consequences are being carefully weighed.

One school of thought advocates widespread use of hormone-replacement therapy to combat osteoporosis, a rapidly increasing cause of morbidity and mortality in elderly women. This school emphasizes the potential advantages of hormone-replacement therapy in reducing the risk of coronary artery disease in later life. Apart from its long term benefits, hormone-replacement therapy can provide obvious and sometimes dramatic relief from transitional symptoms of menopause such as hot flushes. Against such benefits are potential risks of cancer in the endometrium and breast. Such risks are difficult to evaluate, seem to be small and yet generate much concern.

A meeting on the menopause was held at the National Institutes of Health at Bethesda in May 1988. This volume reports the proceedings of that meeting, and is a useful, up-to-date overview. Its main emphasis is on the physical aspects, with good contributions on osteoporosis, cardiovascular implications and breast cancer. The intriguing evidence from animal studies for the effects of oestrogens in accelerating ovarian ageing is well

reviewed, and some useful light is thrown on the unknown nature of vasomotor flushes.

One of the most controversial issues on menopause is its relation to mental health, in particular depression. The weight of evidence has suggested that whereas the transition through the menopause is not associated with any substantial increase in depressive illness, many women find themselves less able to cope with life's problems so that there is an overall modest increase in psychological morbidity. Two chapters report new data on this issue. The McKinleys carried out a longitudinal study in the United States of women passing through the menopause. These data are limited by the authors' reliance on telephone interviews, but the authors conclude that the menopause has no noticeable impact on mental health. Andrew Whitehead's British study further confirms the earlier picture of a modest increase in morbidity.

One issue which has not been addressed by either of these studies, or others before them, is the possibility that in a proportion of women falling levels of oestrogen after the menopause may contribute to depression whereas for others there may actually be an improvement in mental health and a reduced likelihood of depression, possibly because of their escape from the negative effects of the menstrual cycle. These two contrary effects of the menopause conform with clinical impressions. If they do exist, and are not studied separately, they could combine to produce a picture of no effect.

Those working in the field as well as those interested in struggling to assess the risks and benefits of hormone-replacement therapy will find this book a useful addition to the literature. □

John Bancroft is at the MRC Reproductive Biology Unit, Chalmers Street, Edinburgh EH3 9EW, UK.

Sticking to the facts

Martin J. Humphries

Fibronectins. By Richard O. Hynes. *Springer-Verlag: 1990. Pp. 546. £92, \$129.*

MANY of the recent key advances in our understanding of the mechanisms of cell-matrix adhesion have come either directly or indirectly from work on the glycoprotein fibronectin. These include the amino-acid sequence data that gave insight into the modular nature of large extracellular proteins, the discovery of the RGD adhesive recognition signal peptide, the identification of alternatively spliced domains with functional significance and, most recently, identification of the integrin family of adhesion receptors. These, and related areas, have been reviewed over the past few years. But to place such topical areas of research interest in context, it is occasionally necessary to view them against the scaffold of accumulated knowledge from which they arose. The main strength of *Fibronectins* is that it provides a complete, up-to-date treatise on the cell biology, biochemistry and molecular biology of these fascinating molecules.

In his introduction to the book, Richard Hynes is reluctant to present chronological data on the number of publications on fibronectin, but notes that the rate is now sufficiently high to deter anyone from starting to compile a comprehensive review of the protein. The fact that he has achieved this goal is a testament to his scholarship. It is inevitable in any active research area that a review, never mind a book, is somewhat out of date before publication. However, to quote the preface, "the literature up to the end of 1988 is reviewed thoroughly and completely . . . and major advances occurring up to the time of final copyediting (August 1989) are included". Nothing better could be expected.

The book is based broadly on the classical framework for a review; it has a brief, though always fascinating, historical perspective to the field, followed by a technically useful chapter on methodology, and then describes fibronectin distribution (*in vivo* and *in vitro*), its structure, its molecular interactions, and current views of its functions in embryonic development, oncogenic transformation, haemostasis, wound-healing, inflammation and microbial infection. Finally, there is a necessarily personal perspective of areas for future exploitation. I found the index a little lacking in detail, but at the same time had no real problem locating specific information. Perhaps someone less used to

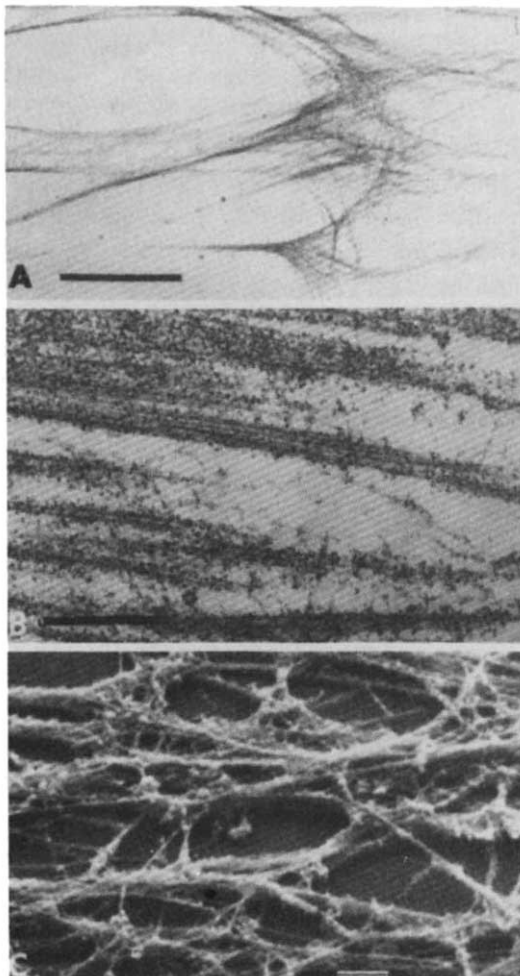
fibronectin would have slightly more trouble.

The book reads more like a detailed journal review than a scientific novel. For this reason, it would be wise to discuss its future readership. For those already addicted to fibronectin, and for workers new to the area, it will be an invaluable reference text. In general, other previous landmark monographs on this subject, such as the volume *Fibroblast Surface Protein* (Annals of the New York Academy of Science, 1978) and the recent book *Fibronectin* edited by Deane Mosher (Academic) have been collections of reviews contributed by several authors with particular expertise. Although providing excellent synopses of selected areas, there was, inevitably, overlap and a lack of continuity between chapters. Hynes is the first person to attempt to review the whole field single-handedly, and the book benefits in these areas as a result. Furthermore, because of his wide knowledge of the field and his contributions to many aspects of fibronectin research, there are no weak chapters. Approximately one-third of all papers on fibronectin, and therefore the overwhelming majority of those thought to be significant, are cited in the impressive bibliography (more than 2,000 references). Every aspect of fibronectin structure and function is considered in detail.

For workers in related areas, the book will also be informative, particularly because of the prototype nature of fibronectin research. I suspect that cell biologists peripheral to this area may find the book somewhat esoteric, although it will almost certainly be able to fulfil their requirements. The text is specific, but eminently readable, the facts being interspersed with brief personal comments and viewpoints. Despite Hynes's enormous contribution to fibronectin research, he has not produced a dogmatic monograph, but a fair reflection of the productivity of several hundred laboratories. I looked for speculation and controversial opinion, but Richard has apparently steered himself to introduce as little as possible. Those people who pick up this book looking for a personal view will be only partially satisfied, but anyone interested in the fascinating history of fibronectin research, a comprehensive factual survey of the field and a staccato smattering of personal insight, will be sated.

The main highlights for me are the numerous full-page tables of information painstakingly gleaned from the literature,

and the careful treatment of several difficult and controversial areas. The only slight weakness is that the book is dominated by fibronectin. Such a statement may seem ludicrous for a book with that title, but it is becoming clear that an understanding of the relevance and regulation of cell adhesion requires an



Fibronectin-rich matrices: (A) TEM, bar 0.5 μm ; (B) immunoferritin labelling, bar 0.5 μm ; (C) SEM, bar 15 μm . (From *Fibronectins* — first published by L. B. Chen *et al.* in *Cell* **14**, 377–391; 1978.)

appreciation of the contribution of other matrix molecules and receptors. As a review of fibronectin, the book is close to perfect, but for most of the subjects it covers there is substantial evidence implicating other molecules in the same events, and therefore the role of fibronectin is not placed in its proper context. Unfortunately, to do so another 2,000 references would probably have been needed and Hynes would have given up long ago. Having said this, the overall directive for the series of monographs of which this is a part is to provide "a concise assessment of the state of a subject in a well-defined field". This is achieved successfully. □

Martin J. Humphries is in the Department of Biochemistry and Molecular Biology, University of Manchester, Oxford Road, Manchester M13 9PT, UK.

Light show

Peter Knight

Laser Science and Technology: An International Handbook. Volumes 1–6*. Series editors V.S. Letokhov, C.V. Shank, Y.R. Shen and H. Walther. Harwood: 1989.

IN ANY active field such as laser science, the reporting of new discoveries, their incorporation into the accepted body of knowledge and their diffusion into other nearby fields or into teaching texts is rather haphazard. The intermediate step between research paper and textbook was traditionally the review article couched in language accessible to a wide audience. But such reviews are increasingly rare, and busy researchers are unlikely to spare the time to write monographs. So where does a newcomer turn to find a balanced and readable account of contemporary developments?

Harwood have issued a multipart handbook to cover both fundamental and applied aspects of lasers and quantum electronics. Each part is separately issued as a paperback, typically between 50 and 100 pages in length, and each is designed as a self-contained review from the basics to current developments. Volumes that have appeared, or are in preparation, cover basic physics (squeezed light, multiphoton physics and chaos in non-linear optics), systems (distributed feedback lasers and excimer lasers, for example), and scientific and engineering applications.

Of the six under consideration here, some are hardly distinguishable from conventional specialist review articles produced by established journals, whereas others do seem to offer accessible and useful guides for the novice. Each article is typeset, which is a relief from the unpleasant camera-ready format adopted elsewhere. But many of the figures are so badly reproduced as to be useless: broken type, blurs from the use of inadequate photocopies, hand-lettering, paste-ups at crazy angles . . . such amateurishness is unwarranted for the price. I hope the editors can change such publishing laxity; after all, they are claiming that their series is more accessible than standard reviews.

If the work of the publisher is dis-

- *1. *Laser Microirradiation of Cells* By T. Kasuya and M. Tsukakoshi. Pp. 80. \$38, £23.
2. *Nonlinear Optical Diagnostics of Laser-Excited Semiconductor Surfaces* By S.A. Akhmanov, N.I. Koroteev and I.L. Shuman. Pp. 76. \$37, £22.
3. *Light Pulse Compression* By Wolfgang Rudolph and Bernd Wilhelmi. Pp. 132. \$59, £36.
4. *Analytical Aspects of Atomic Laser Spectrochemistry* By Kay Miemax. Pp. 44. \$23, £14.
5. *Investigation of Short-Lived Isotopes by Laser Spectroscopy* By Ernest W. Otten. Pp. 76. \$38, £23.
6. *Nonlinear Effects in Optical Fibers* By E.H. Dianov, P.V. Mamyshev, A.M. Prokhorov and V.N. Serkin. Pp. 58. \$32, £19.

appointing, the authors have done much better. The two related articles by Rudolph and Wilhelmi, and by Dianov *et al.*, present a splendid account of ultrashort pulse compression and optical solitons. On balance, this looks like a good series which will certainly be used as a source of reference material once the production difficulties are overcome. □

Peter Knight is in the Blackett Laboratory, Imperial College, London SW7 2BZ, UK.

Into the shadows

Robert N. Proctor

Health, Race and German Politics between National Unification and Nazism 1870–1945. By Paul Weindling. Cambridge University Press: 1989. Pp. 641. £55, \$69.50.

IN recent years there has been an explosion of historical writing on science and medical policy under the Nazis. Paul Weindling's new book adds substantially to this literature, tracing the growth of German eugenics from the utopian social darwinism of the 1890s to the criminal medicine of the Nazis. Based on extensive research in East and West German archives, Weindling shows that the rise of German eugenics must be understood in the context of public-health and welfare institutions, and not simply as an offshoot of Aryan supremacist ideology.

Many of these themes are familiar, but much new detail is added: on Ploetz's private antisemitism, on publisher J. F. Lehmann's *Freikorps* activity, and on the fears of venereal disease that generated Ibsen's *Ghosts*. Weindling describes Haeckel's hierarchical organicism, Forel's early experiments with castration, Voronoff's experiments transplanting monkey testicles (into humans for the purposes of rejuvenation), and the early anti-alcohol movement. His account mixes the ominous and the downright silly, as we hear not only about early plans for euthanasia, but also about the convictions within Ploetz's circle that, as humans are mammals, they should dress in clothing made from mammalian fibres.

Weindling does an interesting job tracing the appeal to biological metaphors in political life — metaphors of the cell-state and the cell-army, comparisons of plasma to soldiers and of the genome to the general staff. Carl Correns compared hereditary units to machine-gun bullets; others complained about the rising "monopoly of the nucleus" (*Kernmonopol*) in concepts of cellular function. Weindling is sensitive to the changing political fortunes of scientific theories, from the ban in 1879 on evolution in Prus-

sian schools (when biology became "a casualty of the anti-socialist laws") to the Nazi endorsement of Weismann's principle of the immutability of the germ plasm. Biologically based politics are described as a strategy for German national unification, one whose political character changed with the changing political fortunes of nationalism.

The strength of the work is its voluminous detail — Weindling has examined an extraordinary range of sources. This strength is related to the book's primary weakness, insofar as the steady stream of details sometimes seems haphazardly organized, with insufficient attention to matters of style. We hear about public-health reformers developing "an institutional infrastructure in associations and the state for the provision of welfare", and similar convolutions. The text is further dulled by excessive use of the passive voice ("a surge in population growth meant that a range of political structures and values were resented as restrictive") and occasional repetitions.

If there is an overarching theme to the work, it is that an "ideology of professional expertise" helped to bring about the authoritarian medicine we associate with the Nazi era. Science and medicine "served to define the social status of intellectual elites", as eugenics ideals merged with the goals of the nascent welfare state. The Weimar republic was unstable because technocratic professionals erected antidemocratic and coercive social structures.

At times, I found myself uneasy with the repeated allusions to the "rule of professional elites". (After all, there have been other states with equally strong professional elites, where fascism was not the consequence.) Weindling suggests that eugenics was a barrier to democratization because "its scientific claims required professional expertise". On such criteria most medicine — and probably most science — must be judged antidemocratic. Eugenics was generally hostile to democracy, not because it was promoted by professionals, but rather because it posited a hierarchy of races and/or lives judged worthy or unworthy of living. The fact that eugenicists were professionals (most were physicians) aided them in achieving their goals, but it did not exhaustively define their politics. Professionals, after all, were also involved in resistance to eugenics and to fascism — as Weindling takes pains to illustrate. Professionalism and democracy (or science and democracy) are not inherent opposites.

Weindling distinguishes between technocrats and ideologues in the racial hygiene movement. Ideologues championed racial purity and a romantic return to the past; technocrats championed professional power and "value-neutral" science. If there is a salient feature of Nazi

racial hygiene, however, it is that the boundary between technocrats and ideologues was not a fast one. Was Otmar von Verschuer an ideologue or a technocrat? The racist rhetoric of Lenz or Fischer or Verschuer was certainly as odious as that of Streicher or Wagner; the fact that it was clothed in science did not make it any less ideological. Postwar denazification officials pondered whether men such as Verschuer should be considered "racial fanatics" or "dedicated scientists"; few recognized that it was possible to have been both. The American mentioned by Nachtsheim (and cited by Weindling) who stated that anthropologists were "1,000 times more guilty than the average idiotic SS man" was probably not far from the truth.

In a volume of such a scope there are bound to be a few errors: J. F. Lehmann, the notorious publisher of racial hygiene (whose name now adorns every bookstore of the West German Medical Association), joined the Nazi party in 1920, not in 1932 as Weindling states. This qualified Lehmann to receive the very first *Goldene Ehrenzeichen*, an honour bestowed only upon the first 100,000 members of the party. Weindling elsewhere states that most racial hygienists remained "aloof" from the Nazi party, supporting this with statistics to the effect that only one in four members of the Munich Society for Racial Hygiene had joined the party by January 1933, before Hitler's rise to power. This is hardly evidence of apathy. In attempting to rectify the traditional assumption linking eugenics exclusively with the political right, Weindling goes a bit too far in the opposite direction, characterizing eugenics as a "central focus" of the Socialist Doctors Association. A few socialists supported eugenics, but not to the extent that parties of the right did (with the notable exception of Catholics).

Weindling's is not likely to be a popular book; it is not designed to be. But he has clearly helped to illuminate the shadows that historians of medicine have tended to avoid in their focus on the brighter side of their subject. □

Robert N. Proctor is at the New School for Social Research, New York, New York 10011, USA.

■ R. N. Proctor's book *Racial Hygiene: Medicine under the Nazis* has just appeared in paperback. Publisher is Harvard University Press, price £11.95, \$14.95. For review see *Nature* **334**, 573 (1988).

■ To be published next week by Basic Books is *The Genocidal Mentality: Nazi Holocaust and Nuclear Threat*, by psychiatrist R. J. Lifton and sociologist E. Markusen. The authors attempt to show how the demands of professionalism, the pressures of bureaucracy and dissociative processes such as "psychic numbing" enable scientists and strategists to "remain sane in the service of social madness". Price is \$22.95.

Universal control mechanism regulating onset of M-phase

Paul Nurse

The onset of M-phase is regulated by a mechanism common to all eukaryotic cells. Entry into M-phase is determined by activation of the p34^{cdc2} protein kinase which requires p34^{cdc2} dephosphorylation and association with cyclin.

THE cell cycle is the period during which events required for successful cell reproduction are completed. The two major events common to all cell cycles are S-phase¹, when chromosomes are replicated, and M-phase², when the replicated chromosomes are segregated into two daughter cells. In this article I discuss the control regulating the onset of M-phase during the cell cycle. A case can now be made for the existence of a universal control mechanism common to all eukaryotic cells. Central to this mechanism is the protein kinase p34^{cdc2} which is activated at both mitotic and meiotic M-phase. Activation requires changes in phosphorylation state of the kinase and its interaction with cyclins, a class of proteins which vary in level during the cell cycle. The p34^{cdc2} protein kinase is thought to phosphorylate key proteins which lead to the major M-phase events, including chromosome condensation, cytoskeletal reorganization, nuclear envelope breakdown and cell shape changes.

M-phase controls

The onset of M-phase is subject to a number of controls. In many cells there is a tight coupling between increase in cell mass and progress through the cell cycle³. Entry into M-phase takes place when cells attain a critical mass, and the rate at which a cell accumulates mass determines the overall periodicity between cell divisions. In other cells the period between M-phases is determined by a timer or oscillator^{3,4}. This applies to cells in early embryos which undergo repeated divisions with little cell growth. A further control, operative in somatic cells, couples M-phase onset with completion of S-phase^{5,6}. This link ensures that M-phase is delayed if chromosomes are damaged or are not fully replicated. There can also be a direct effect of changes in growth rate on the timing of M-phase, with faster growing cells dividing at a greater mass than slower growing ones^{3,7}. Therefore the controls acting over M-phase can be coupled to mechanisms monitoring time, cell mass, growth rate, and the completion of chromosome replication.

Two approaches have been used to identify components of these controls. In the first, primarily genetic, approach, regulatory genes have been identified which influence the cell cycle timing of M-phase. Only some of the events required for M-phase to take place are important for its timing. These events

can be distinguished because speeding up the rate at which they are completed will significantly advance a cell into M-phase, whilst speeding up other events will have little or no effect on the timing of M-phase (see Fig. 1). This reasoning was the basis for the identification of regulatory genes in the fission yeast *Schizosaccharomyces pombe*; mutants in these genes lead to the advancement of cells into mitosis^{8,9}. In the second, primarily biochemical approach, proteins have been purified which enable *Xenopus* and starfish oocytes, naturally arrested just before meiotic M-phase, to enter meiosis^{4,10}. Similar logic is used in both the genetic and biochemical approaches: mutating genes or adding proteins advances M-phase in cells proceeding through the cell cycle, or enables M-phase to take place in cells naturally arrested in the cell cycle.

Regulatory genes in yeast

The controls regulating mitotic onset in fission yeast involve at least four gene functions acting together in a network (Fig. 2) (refs 11–14). The network comprises two independent pathways which act through the *cdc2*⁺ gene product p34^{cdc2}. Antibodies raised against p34^{cdc2} immunoprecipitate a 34K (*M_r* 34,000) protein kinase activity¹⁵. The *cdc2*⁺ gene¹⁶, like its budding yeast equivalent *CDC28* (ref. 17), is also required earlier in the cell cycle at the onset of S-phase^{18,19}, but this aspect of its function falls outside the scope of this review. One pathway of the mitotic regulatory network is inhibitory and includes the *nim1*⁺ and *wee1*⁺ gene products which are probably threonine/serine protein kinases, whereas the other pathway is activatory and includes the *cdc25*⁺ gene product. The balance of these two pathways regulates p34^{cdc2} function and advances or delays onset of mitosis (Fig. 2) (refs 11–14).

Two different types of mutant were used to establish how the network operates: *cdc* mutants, first described in budding yeast¹⁹, delay mitotic onset and lead either to cell cycle arrest²⁰ or to division at an increased cell size⁹; by contrast, *wee* mutants advance cells into mitosis and lead to division at reduced cell size^{8,9}. All four genes in the regulatory network can be mutated or over-expressed to generate a *wee* phenotype^{11–14}. Over-expression of the activators *cdc25*⁺ and *nim1*⁺, or deletion of the inhibitor *wee1*⁺, advances cells into mitosis. The mutations in *cdc2*⁺ giving a *wee* phenotype are more subtle in effect because advancement is brought about not by *cdc2*⁺ over-expression but by dominant missense mutations^{9,21–23} generating a p34^{cdc2} with altered control characteristics. Thus the overall timing of mitosis can be influenced by any one of these four gene products.

The two pathways operate upstream of overall p34^{cdc2} function. This was demonstrated by the existence of mutant forms of p34^{cdc2} which are largely insensitive to changes in activities of the *cdc25*⁺, *wee1*⁺, or *nim1*⁺ gene products^{11–14}. The two pathways act independently, because mutations affecting genes in the two separate pathways, such as *wee1*⁺ and *cdc25*⁺, are additive in their effects. For example, if a cell harbours *wee* mutations in both genes then mitosis is advanced so prematurely that it is lethal. These results are consistent with the scheme²³ controlling p34^{cdc2} activation at mitotic onset illustrated in Fig. 2. Also included on this scheme are the *win* (ref. 24) and *cdr*

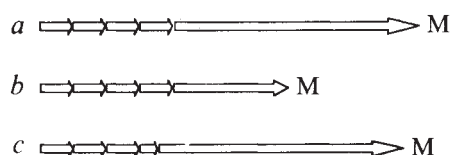


FIG. 1 Events influencing timing. The lengths of the arrows represent the time required for an event to take place. The longer arrow shown in *a* is potentially an event which can influence the timing of M-phase. When it is speeded up, as in *b*, M-phase is considerably advanced. Speeding up one of the events represented by the shorter arrow, as in *c*, has little effect on M-phase timing. Note that blocking an event does not distinguish the two types, as in all cases onset of M-phase will be blocked. For a more sophisticated account of rate determining steps in the context of flux control in metabolic pathways, see ref. 103.

(ref. 25) gene functions which interact with the network, but in a manner yet to be fully established.

Two further gene products also associated with $p34^{cdc2}$ are $p56^{cdc13}$ (ref. 26) (which is also called $p63^{cdc13}$) (ref. 27), and $p13^{suc1}$ (refs 28–31). Allele-specific interactions between $cdc2$ and $cdc13$ mutants and the fact that $cdc2^+$ over-expression suppresses the partial function mutant $cdc13-117$, suggested that these two gene products interact closely³². This was confirmed biochemically by co-immunoprecipitation experiments demonstrating that $p34^{cdc2}$ and $p56^{cdc13}$ form a physical complex²⁷. Figure 2 shows that $p56^{cdc13}$ is required both before and during mitosis (refs 26 and 33).

$p13^{suc1}$ is also physically associated with $p34^{cdc2}$. Specific $suc1$ mutants or over-expression of $suc1^+$ suppress certain $cdc2^{ts}$ alleles^{30,31}, and passage of cell extracts through a column containing $p13^{suc1}$ antibodies depletes both the $p34^{cdc2}$ and $p13^{suc1}$ proteins²⁸. About 5% of the total cellular $p34^{cdc2}$ is thought to be associated with $p13^{suc1}$, and $p13^{suc1}$ bound to Sepharose beads can be used to enrich $p34^{cdc2}$ from cell extracts²⁷. The role of $p13^{suc1}$ at mitosis is not clear (see later), but $suc1^+$ over-expression leads to a delay of mitotic onset^{29,31}, whereas deletion of $suc1^+$ results in cells arresting within mitosis²⁶.

Factors inducing M-phase

Parallel with these genetic studies has been a biochemical approach using various invertebrate and vertebrate oocytes and eggs^{4,10}. Oocytes can be induced to proceed synchronously into meiotic M-phase, and eggs undergo synchronous rounds of mitotic cleavages during early embryo development. Work on the regulation of M-phase using these systems began with the description of maturation-promoting factor (MPF) in *Xenopus*³⁴. Injection of the contents from an egg into an oocyte induced entry into M-phase and maturation into an egg. Levels of similar M-phase-inducing factors oscillate in oocytes and eggs, peaking at M-phase^{35,36}. Various other agents induce maturation, but unlike MPF they require continued protein synthesis for their action^{37,38}. These other agents are thought to operate upstream of the M-phase control and are probably involved in the signal transduction pathway induced by hormones during natural oocyte activation.

Initial preparations of MPF were either crude cellular extracts or partially purified fractions. Recently, MPF has been highly purified from *Xenopus*, and activity found to be associated with two proteins of apparent M_r 32,000 (32K) and 45,000 (45K) (ref. 39). Immunoblotting and immunoprecipitation experiments using antibodies raised against $p34^{cdc2}$, demonstrated that the smaller protein is the *Xenopus* equivalent of $p34^{cdc2}$ (ref. 40). A similar conclusion was reached using $p13^{suc1}$ which

blocked entry into M-phase when added to *Xenopus* extracts⁴¹. Passage of extracts through a column of $p13^{suc1}$ beads depleted the *Xenopus* equivalent of $p34^{cdc2}$ and also depleted MPF activity. Highly purified preparations of a starfish M-phase-specific kinase also had MPF activity and contained the starfish equivalent of $p34^{cdc2}$ (refs 42 and 43). Two starfish purification procedures have been used, one generating a preparation containing only $p34^{cdc2}$, and the other $p34^{cdc2}$ together with a further protein of 47K (M_r 47,000) (refs 42 and 43). These results demonstrate that $p34^{cdc2}$ is a component of MPF in both *Xenopus* and starfish.

The other 45–47K component of MPF is a cyclin. Cyclins were initially described in sea urchin eggs as a class of proteins synthesized during the cell cycle that become destroyed at the end of M-phase^{44,45}. They are present as major translation products of maternal mRNA in various invertebrate and vertebrate eggs and share about 30% identity in sequence over a central region of around 200 amino-acid residues. Two classes, cyclins A and B, can be distinguished which differ slightly in this central region⁴⁵ although the functional significance of these differences is not yet clear.

Involvement of cyclins in regulation of M-phase was initially suggested when injection of clam cyclin A mRNA into *Xenopus* oocytes induced their entry into meiotic M-phase⁴⁶. Further support came from experiments using *Xenopus* extracts which can direct the major events of the cell cycle *in vitro*. Nuclei introduced into these extracts undergo successive S- and M-phases⁴⁷. In this system M-phase was blocked if oligonucleotides were used to direct RNAase specifically to cleave cyclin mRNA⁴⁸. Ablation of all messages with RNAase also blocked M-phase, and this block was reversed after addition of RNAase inhibitor and cyclin message⁴⁷. Therefore the only protein synthesis required for M-phase in these extracts is cyclin synthesis, indicative of an important regulatory role for cyclins. Co-precipitation experiments from clam⁴⁹ extracts suggested that $p34^{cdc2}$ and cyclins could be physically associated, and direct proof that cyclins were part of MPF was obtained when the 47K component of purified starfish MPF was sequenced and found to be a cyclin⁵⁰. The universal significance of cyclins was established by the demonstration that the fission yeast $p56^{cdc13}$ was also a cyclin. The sequence of the $cdc13^+$ gene indicated that it encoded a B-cyclin-like protein^{33,51,52} which, like other cyclins, is destroyed at the end of mitosis^{26,27}. However, it has yet to be shown that $p56^{cdc13}$ from yeast is interchangeable in function with cyclins from other organisms.

$p34^{cdc2}$ and cyclins are also important for regulating M-phase in mammalian cells. The human homologue of $cdc2^+$ was cloned by complementation using a fission yeast $cdc2^{ts}$ mutant strain and a cDNA expression library prepared from human cells⁵³. This procedure uses the yeast mutant cells to select for a human gene with the same biological function as the yeast $cdc2^+$ gene. The human *CDC2* gene can completely substitute for the yeast $cdc2^+$ gene, suggesting that there is considerable overlap in their functions. The human gene encodes a 34K protein, 63% identical in sequence with the yeast gene⁵³. Thus there is remarkable conservation of this gene both structurally and functionally from yeast to humans. The link between yeast and mammals has also been made using antibodies raised against fission yeast $p34^{cdc2}$ which were found specifically to detect a 34K protein kinase in human cells⁵⁴, and by injection of anti- $p34^{cdc2}$ antibodies into rat cells which was found to block cell division⁵⁵. A human cyclin homologue has also been cloned and encodes a protein which is destroyed at the end of mitosis and co-immunoprecipitates with $p34^{cdc2}$ (ref. 56). Given these results it is likely that $p34^{cdc2}$ and cyclins are important for inducing M-phase in all eukaryotic cells.

Activation of $p34^{cdc2}$ protein kinase

The molecular mechanism of M-phase induction involves activation of $p34^{cdc2}$ protein kinase activity. The kinase activity is

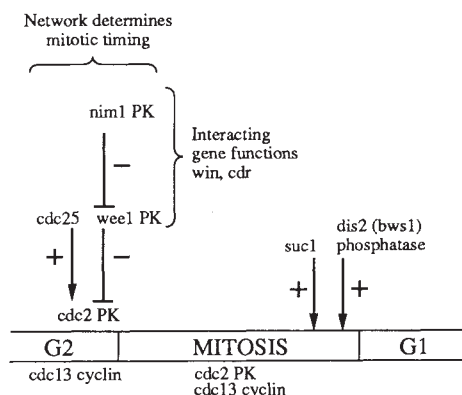


FIG. 2 Regulatory genes in fission yeast. The gene products of $cdc2^+$, $cdc25^+$, $wee1^+$ and $nim1^+$ act in a regulatory gene network which determines the cell-cycle timing of mitosis. $cdc13^+$ is required for mitotic onset and together with $cdc2^+$ is also required later during mitosis. The genes $suc1^+$ and $dis2^+$ (allelic with $bws1^+$) are probably required towards the end of mitosis. The gene products of $cdc2^+$, $wee1^+$ and $nim1^+$ are protein kinases, $dis2^+$ a phosphatase, and $cdc13^+$ a cyclin.

directed against serine and threonine residues in the motif S/TP flanked by basic residues^{57,58}. This motif is well represented in H1 histone which acts as an excellent *in vitro* substrate. Kinase activity is high in proliferating yeast and mammalian cells but is much reduced in cells withdrawn from the cell cycle^{15,57,58}. Activity rises to a peak during M-phase and falls to a low level during interphase in *Physarum*^{59,60}, starfish⁴², *Xenopus*⁶¹, mammalian cells⁶² and fission yeast^{26,27}. The *Physarum* studies^{59,60} preceded the other work by nearly 15 years and it was also shown that addition of the kinase to the *Physarum* plasmodium advanced mitosis^{60,63}. However, at this time it was not known that the kinase was encoded by *cdc2*⁺, although the possibility was suggested⁶⁴.

Activation of the kinase activity at M-phase is correlated with p34^{cdc2} dephosphorylation in starfish⁴³, *Xenopus*⁶¹, mammalian cells⁶⁵ and fission yeast⁶⁶. Phosphorylated threonine and tyrosine⁶⁷ residues in p34^{cdc2} both become dephosphorylated^{65,66,68}. Kinase activation is accelerated in starfish extracts when conditions favour phosphatase activity⁴³, and is inhibited in *Xenopus* extracts by addition of p13^{suc1}, which prevents dephosphorylation of phosphotyrosine⁶⁸. In fission yeast, the phosphorylated tyrosine is located in the ATP-binding site of p34^{cdc2} (ref. 66). Mutating this residue to a phenylalanine (which prevents phosphorylation) activates p34^{cdc2} function and leads to advancement of cells into mitosis.

These results suggest a simple model whereby p34^{cdc2} kinase activity is inhibited during interphase when this tyrosine is phosphorylated because ATP binding or use is blocked. At the G2/M boundary this phosphate group is removed allowing kinase activity to appear, resulting in entry into M-phase. DNA sequences of the genes reveal that a tyrosine is found in the ATP-binding site of all prospective *cdc2*⁺-like proteins known so far, and so this model may apply to all eukaryotes⁶⁶. It has been suggested⁶⁷ that mammalian p34^{cdc2} is phosphorylated on tyrosine *in vivo* by p60^{src}, but the physiological relevance of this is not clear given that the phosphorylation is likely to inhibit p34^{cdc2} and thus inhibit division, an effect not expected from the action of a proto-oncogene. It is also unlikely that *wee1*⁺ is responsible for the tyrosine phosphorylation given that it is thought to encode a threonine/serine protein kinase^{12,13}.

In fission yeast the *cdc25*⁺ activator is required for p34^{cdc2} kinase activation^{26,27}. In a *cdc25*^{ts} mutant incubated at the restrictive temperature, p34^{cdc2} is phosphorylated⁶⁶ and its kinase activity is low²⁶. Within minutes of shifting to the permissive temperature, p34^{cdc2} becomes dephosphorylated⁶⁶ and kinase activity appears²⁶. The *cdc25*⁺ gene encodes a 80K phosphoprotein⁶⁹ but the molecular basis of its action is unknown as its sequence has no similarities with proteins of known biochemical function¹². Given that its function leads to p34^{cdc2} dephosphorylation, it is likely that *cdc25*⁺ either inhibits a kinase acting on p34^{cdc2} or activates a phosphatase acting on p34^{cdc2} (ref. 66). The level of p80^{cdc25} increases as cells proceed through G2, reaching a peak just before the onset of mitosis⁶⁹. This result, together with the observation that increased levels of p80^{cdc25} advance cells into mitosis¹², suggests that the accumulation of p80^{cdc25} to a critical level in the cell has a key role in determining the cell-cycle timing of p34^{cdc2} kinase activation and thus of mitotic onset.

In extracts of *Xenopus* eggs, activation of the p34^{cdc2} kinase requires both soluble and particulate material⁷⁰. There is also evidence that MPF activation is autocatalytic. For example, amplification of MPF levels in *Xenopus* oocyte extracts can be achieved by addition of small amounts of partially purified MPF (ref. 71). Such an autocatalytic mechanism may be important for bringing about the steep rise in p34^{cdc2} kinase activity which occurs at the onset of M-phase and ensures that the transition between the interphase and M-phase states is rapidly completed. The molecular mechanism of this autocatalysis and its relationship to other components of the activating process are not known but it is thought to involve phosphorylation⁷¹.

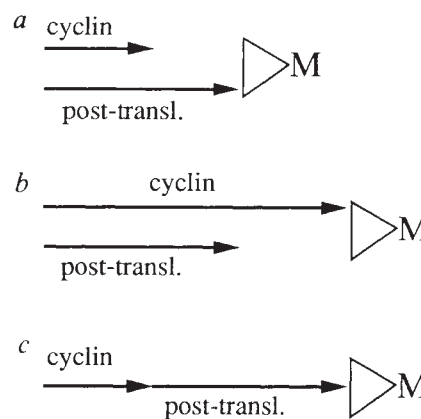


FIG. 3 Cyclin role in M-phase timing. The lengths of the arrows represent the times required for cyclin synthesis to lead to sufficient cyclin for M-phase to take place and for the post-translational events required to be completed. *a*, Necessary cyclin synthesis is completed early (although cyclin synthesis still continues) and M-phase timing is determined by the post-translational steps. *b*, The situation is reversed and cyclin synthesis now determines M-phase timing. *c*, The processes are sequential and both contribute to timing. Both *a* and *c* would explain the observed results when protein synthesis is inhibited, although only in case *c* does cyclin synthesis play a part in M-phase timing.

Role of cyclins in activation

Cyclins are required for p34^{cdc2} kinase activation; deletion of *cdc13*⁺ in fission yeast prevents kinase activation at mitosis²⁶, and destruction of cyclin mRNA in *Xenopus* extracts prevents the appearance of MPF activity^{47,48}. Cyclins also have a continuing role during M-phase after activation. The fission yeast partial-function mutant *cdc13-117* allows p34^{cdc2} kinase activation²⁶ but only some aspects of mitosis take place³³. Chromosomes become condensed but the interphase microtubular cytoskeleton does not reorganize into a mitotic spindle.

In *Xenopus*, deletion of part of the cyclin gene generates a mutant protein which allows entry into M-phase in oocyte extracts but not its completion⁷². As noted earlier, p34^{cdc2} is physically associated with cyclin. These data suggest that *in vivo*, the active kinase in MPF is a complex between these two proteins which is required both to initiate M-phase and during M-phase. Highly purified p34^{cdc2} can have *in vitro* kinase activity without being associated with cyclin⁴³, probably because cyclin becomes degraded or dissociated during purification, but such material may not fully reflect the activities of p34^{cdc2} *in vivo*. Other proposed roles for cyclins are to modify p34^{cdc2} kinase specificity^{45,49} and to promote transfer of p34^{cdc2} into the nucleus²⁷.

Cyclins are clearly required for M-phase, but does their accumulation to a critical level determine the cell-cycle timing of M-phase? In fission yeast, unlike the situation for p80^{cdc25} described above, there is no evidence that p56^{cdc13} has such a role²⁶. The patterns of cyclin accumulation in *Drosophila* embryos also suggests that cyclins do not determine the timing of mitosis^{73,74}. In *Xenopus* the situation is more complex. Cyclins are the only proteins which need to be synthesized each cell cycle^{47,48}, but if inhibitors of protein synthesis are injected into eggs during the second half of the cell cycle, M-phase can still take place⁷⁵. Similar observations have been made *in vitro* using cycling extracts⁴⁸. These results suggest that the requirement for cyclin synthesis is completed during the first half of the cycle, some time before onset of M-phase⁷⁵. Two possible explanations of these results are illustrated in Fig. 3*a-c*. The process of cyclin synthesis may have to be completed before a series of post-translational events is initiated. Completion of these events then leads to p34^{cdc2} activation (Fig. 3*c*). Cyclin synthesis and the post-translational events occur one after the other and generate

a single pathway which determines the cell-cycle timing of M-phase. Thus, cyclin synthesis contributes to the timing but this contribution is made early in the cycle. Alternatively, the process of cyclin synthesis acts in parallel with the series of post-translational events, to generate two pathways, both of which need to be completed before onset of M-phase (Figs 3a and b). With this view, the period of cyclin synthesis can determine M-phase timing, as shown in Fig. 3b, or not, as shown in Fig. 3a. In the latter case cyclin synthesis occurs early in the cycle but does not contribute to the cell-cycle timing of M-phase, which is determined entirely by the other pathway. At present the possibilities illustrated on Fig. 3a and c cannot be distinguished.

Mechanism of M-phase onset and exit

Once the p34^{cdc2} kinase is activated it brings about the various events of M-phase including chromosome condensation, cytoskeletal reorganization, nuclear envelope breakdown and cell shape changes, although not all these events occur in all eukaryotes. Understanding the mechanism by which these events are brought about requires identification of the *in vivo* substrates of the p34^{cdc2} kinase. A number of proteins have been shown to be phosphorylated *in vitro* by p34^{cdc2}, including H1 histone^{42,57-60}, p40 (refs 18 and 76), p60^{src} (refs 77 and 78), RNA polymerase II (ref. 79), T antigen⁸⁰, elongation factor⁸¹, cyclin^{37,39}, and lamins (M. Peter; J. Nakagawa, M. Doree, J. Labbe and E. Nigg, personal communication), and yet more proteins contain sequences similar to the substrate motif S/TP flanked by basic residues⁸². All of these may not be *in vivo* substrates. The p34^{cdc2} is essentially a protein kinase catalytic core and may behave rather promiscuously *in vitro*. Also, there are other genes that are very similar to *cdc2*⁺ in size and sequence^{21,83}, and so it is possible that p34^{cdc2} preparations may contain other closely related protein kinases. Despite these difficulties, some comments can be made about possible roles of these potential substrates in the mechanism of M-phase onset.

It has been suggested that phosphorylation of the mitotic-specific sites of p60^{src} may influence the cytoskeleton, bringing about cell shape changes during mitosis^{77,78}, and that phosphorylation of H1 histone may be important for chromosome condensation^{59,60,63}. It is noteworthy that a number of the potential substrates are chromatin-associated proteins and that the substrate motif may be a DNA-binding unit⁸² (S. Moreno, personal communication). It is possible that such proteins need to be displaced from chromatin to allow chromosome condensation to take place, and that this displacement may be brought about by their phosphorylation. If this is the case it may be misleading to look for links between p34^{cdc2} and the physiological function of these proteins. The reported phosphorylation of lamins by purified starfish p34^{cdc2} (M. Peter *et al.*, personal communication) is of great interest given that lamin phosphorylation is believed to cause disassembly of the nuclear lamina and thus would contribute to nuclear envelope breakdown during M-phase.

Studies of p34^{cdc2} localization in the cell have also been relevant to understanding its role in M-phase onset. An immunofluorescence and cell fractionation study of mammalian cells has indicated that p34^{cdc2} is located in both nucleus and cytoplasm⁸⁵, although other immunofluorescence studies have proposed either solely nuclear⁵⁵, or mainly cytoplasmic⁸⁶ locations. More relevant to the question of substrates is the observation that some of p34^{cdc2} is associated with centrosomes^{55,85}, the staining of which increases during G2 and at mitotic onset⁸⁵. Perhaps phosphorylation of centrosomal proteins by the p34^{cdc2} kinase could have a role in the reorganization of the microtubular cytoskeleton at mitosis.

It is possible that the cell is kept in an M-phase state by high p34^{cdc2} kinase activity. Protein phosphorylation is generally increased during M-phase, which could be a direct consequence of the p34^{cdc2} kinase or an indirect consequence of p34^{cdc2}

activating other protein kinases. Re-entry into the interphase state would require dephosphorylation of these proteins, involving p34^{cdc2} kinase inactivation and phosphatase action. In both fission yeast^{87,88} and *Aspergillus nidulans*⁸⁹, genes encoding prospective protein phosphatases have been identified, which are required in late mitosis. These genes, *dis2*⁺/*bws1*⁺ in yeast and *bimG* in *Aspergillus*, could function by removing the phosphates from the substrates of p34^{cdc2}. The process by which the p34^{cdc2} kinase is inactivated at the end of M-phase is not understood. In principle, rephosphorylation of the ATP-binding site could be responsible but in mammalian cells⁶⁵ kinase inactivation occurs long before rephosphorylation of tyrosine residues in p34^{cdc2}. A good candidate for kinase inactivation is cyclin degradation which occurs as cells exit from mitosis; disruption of the p34^{cdc2} cyclin complex could potentiate p34^{cdc2} kinase inactivation. Another possibility involves p13^{suc1} which is known to associate physically with p34^{cdc2}. When *suc1*⁺ is deleted from fission yeast cells, exit from mitosis is blocked and the p34^{cdc2} kinase is not inactivated²⁶. However, this is a contentious suggestion, because several different roles have been proposed for p13^{suc1}, including both positive²⁸ and negative⁶⁸ effects on kinase activation at M-phase onset. Finally, in immunofluorescent studies of mammalian cells, p34^{cdc2} is found to be associated with membrane vesicles at the end of mitosis⁸⁵, and so a shift between cell compartments may also have a role in kinase inactivation.

Universal control mechanism

These studies support the view that the control mechanism regulating M-phase onset is common to all eukaryotic cells⁹⁰. The likely universal features of this control are summarized in Fig. 4 and below.

(1) Central to the mechanism is the p34^{cdc2} protein kinase, activation of which induces M-phase. The kinase is thought to phosphorylate key proteins which lead to the major events of M-phase. High p34^{cdc2} kinase activity maintains the cell in the M-phase state, and exit from M-phase requires kinase inactivation and phosphatase action.

(2) p34^{cdc2} forms a complex with the second universal component of the mechanism, the cyclins. Cyclin is required for activation of the p34^{cdc2} kinase, and is degraded as cells exit M-phase, which may contribute to p34^{cdc2} kinase inactivation. There are various forms of cyclin but the functional significance of these differences is not clear.

(3) Activation of the p34^{cdc2} kinase is associated with dephosphorylation of phosphotyrosine and phosphothreonine residues in the protein. The phosphotyrosine is located in the ATP-binding site, suggesting that part of the activation mechanism

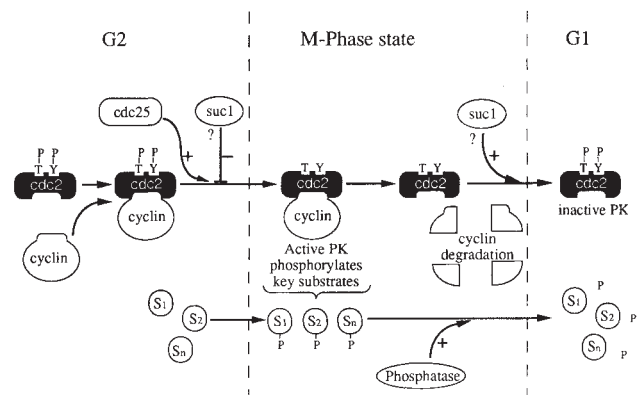


FIG. 4 Universal M-phase control mechanism. The precise order and timing of some of these events such as the relationship between p34^{cdc2} phosphorylation changes and cyclin, *cdc25*⁺ and *suc1*⁺ function are unknown at present. The key substrates could include H1 histone, p60^{src}, lamins, centrosomal proteins, and other proteins which need to be displaced from chromatin to allow chromosome condensation (see text for details).

involves a change at this site allowing the kinase to use ATP.

(4) The timing of mitosis during the cell cycle of fission yeast is determined by a regulatory gene network including genes encoding two further prospective protein kinases, and the *cdc25⁺* gene. *p80^{cdc25}* is required for *p34^{cdc2}* dephosphorylation and kinase activation. Genes sharing some sequence similarities with *cdc25⁺* and having roles in the control of mitosis have been discovered in budding yeast (*MIH1*) (ref. 91) and *Drosophila* (*string*)^{74,92}, suggesting that this element may also be of universal significance.

(5) *p13^{suc1}* interacts closely with *p34^{cdc2}* and may have roles in inhibiting kinase activation or promoting kinase inactivation. It is found in a range of eukaryotes including yeast and human cells.

Coupling the mechanism to controls

Some progress has been made in establishing how this mechanism is coupled to completion of chromosome replication, one of the major controls discussed at the beginning of this review. In fission yeast, blocking DNA replication prevents initiation of mitosis. However, in mutants of *cdc2* which do not require *cdc25⁺*, for function, or in strains which contain high levels of *p80^{cdc25}*, blocking DNA replication does not prevent initiation of mitosis⁹³. In these mutant strains cells attempt to undergo mitosis, but because the chromosomes are not fully replicated the nucleus cannot divide and usually becomes cut by the dividing cell. This indicates that the link between S-phase and mitosis is mediated through *p80^{cdc25}* acting on *p34^{cdc2}* (ref. 93). A number of other genes have been identified in budding yeast^{6,94}, *A. nidulans*^{95,96} and hamster cells⁹⁷, mutation of which allow mitosis to proceed in the absence of correctly replicated chromosomes. However, it is not known if any of their gene products interact with *p34^{cdc2}* or a *p80^{cdc25}* homologue to provide the link between S-phase and mitosis in these organisms.

The involvement of *p80^{cdc25}* in the link between S-phase and mitosis may partly explain the apparent differences between somatic and embryonic cell cycles concerning 'feedback' or 'checkpoint' controls^{6,38} which generate the dependency relations within the cell cycle. In cells of early embryos, unlike most other cells, blocking DNA replication does not prevent aspects of the subsequent mitosis from taking place^{98,99}. In the *Drosophila* embryo there are maternal stores of *string*, the gene product with functional and structural similarity to *p80^{cdc25}*, which are not depleted until around the 14th cleavage^{74,92}. The situation in the early *Drosophila* embryo may be analogous to a fission yeast cell containing high levels of *p80^{cdc25}*. In both cases the dependency of mitosis upon the previous S-phase is not operative because the high levels of *string* or *p80^{cdc25}* uncouple mitosis from DNA replication. If this interpretation is correct then it demonstrates how cell cycles that are apparently regulated in quite different ways can be controlled by the same basic mechanism, the only difference between the two cases being that different control steps are rate limiting (see Figs 1 and 3). In somatic cells the level of *p80^{cdc25}* or its homologue is limiting thus establishing a dependency link between S-phase and mitosis, whereas in embryonic cells the level is high and not limiting, which leads to a breakdown of the dependency link.

It is not yet clear how the M-phase control mechanism is coupled to the other controls monitoring time, cell mass and growth rate, but the requirement to input several signals may explain why there are a number of different elements involved in the mechanism. Various models have been proposed for monitoring time and mass during the cell cycle. For example, time could be monitored by a sequence of events which takes a fixed time to complete^{3,19} or by a limit-cycle oscillator¹⁰⁰. Mechanisms for monitoring cell mass include models in which a fixed amount of inhibitor is diluted out by cell growth or an accumulating activator is titrated out on a fixed number of sites within the cell^{3,101}. A prime candidate for the latter are chromosomal sites which are present in a fixed number in the cell,

accounting for the correlation of ploidy with cell mass¹⁰². It will now be important to investigate whether the regulatory circuits controlling onset of M-phase share any characteristics with these models.

Note added in proof: A homologue of *p34^{cdc2}* has recently been reported to be present in algal and higher plant cells¹⁰⁴. □

Paul Nurse is in the ICRF Cell Cycle Group, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

- Laskey, R., Fairman, M. & Blow, J. *Science* **246**, 609–613 (1989).
- McIntosh, J. & Koonce, M. *Science* **246**, 622–628 (1989).
- Fantes, P. & Nurse, P. in *The Cell Cycle* (ed. John, P.) 11–33 (Cambridge University Press, 1981).
- Kirschner, M., Newport, J. & Gerhart, J. *TIG* **1**, 41–47 (1985).
- Mitchison, J. M. in *The Biology of the Cell Cycle* p. 313 (Cambridge University Press, Cambridge, 1971).
- Hartwell, L. & Weinert, T. *Science* **246**, 629–634 (1989).
- Fantes, P. & Nurse, P. *Exptl. Cell Res.* **107**, 377–386 (1977).
- Nurse, P. *Nature* **256**, 547–551 (1975).
- Nurse, P. & Thuriaux, P. *Genetics* **96**, 627–637 (1980).
- Lohka, M. J. *J. Cell Sci.* **92**, 131–135 (1989).
- Fantes, P. *Nature* **279**, 428–430 (1979).
- Russell, P. & Nurse, P. *Cell* **45**, 145–153 (1986).
- Russell, P. & Nurse, P. *Cell* **49**, 559–567 (1987).
- Russell, P. & Nurse, P. *Cell* **49**, 569–576 (1987).
- Simanis, V. & Nurse, P. *Cell* **45**, 261–268 (1986).
- Nurse, P. & Bissett, Y. *Nature* **292**, 558–560 (1981).
- Beach, D., Durkacz, B. & Nurse, P. *Nature* **300**, 706–709 (1982).
- Mendenhall, M. D., Jones, C. A. & Reed, S. I. *Cell* **50**, 927–935 (1987).
- Pringle, J. R. & Hartwell, L. H. in *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance* (eds Strathern, J. N., Jones, E. W. & Broach, J. R.) 97–142 (Cold Spring Harbor Laboratory, New York, 1981).
- Nurse, P., Thuriaux, P. & Nasmyth, P. *Molec. gen. Genet.* **146**, 167–178 (1976).
- Carr, A. M., MacNeill, S. A., Hayles, J. & Nurse, P. *Molec. gen. Genet.* **218**, 41–49 (1989).
- Booher, R. & Beach, D. H. *Molec. cell. Biol.* **6**, 3523–3530 (1986).
- MacNeill, S. A. & Nurse, P. *Curr. Genet.* **16**, 1–6 (1989).
- Ogden, J. E. & Fantes, P. A. *Curr. Genet.* **10**, 509–514 (1986).
- Young, P. & Fantes, P. *J. Cell Sci.* **88**, 295–304 (1987).
- Moreno, S., Hayles, J. & Nurse, P. *Cell* **58**, (1989).
- Booher, R. N., Alfa, C. E., Hyams, J. S. & Beach, D. H. *Cell* **58**, 485–497 (1989).
- Brizuela, L., Draetta, G. & Beach, D. *EMBO J.* **6**, 3507–3514 (1987).
- Hindley, J., Phear, G., Stein, M. & Beach, D. *Molec. cell. Biol.* **7**, 504–511 (1987).
- Hayles, J., Beach, D., Durkacz, B. & Nurse, P. *Molec. gen. Genet.* **202**, 291–293 (1986).
- Hayles, J., Aves, S. & Nurse, P. *EMBO J.* **5**, 3373–3379 (1986).
- Booher, R. & Beach, D. *EMBO J.* **6**, 3441–3447 (1987).
- Hagan, I., Hayles, J. & Nurse, P. *J. Cell Sci.* **91**, 587–595 (1988).
- Masui, Y. & Markert, C. *J. exp. Zool.* **177**, 129–146 (1971).
- Wasserman, W. & Smith, L. *J. Cell Biol.* **78**, R15–R22 (1978).
- Gerhart, J., Wu, M. & Kirschner, M. *J. Cell Biol.* **98**, 1247–1255 (1984).
- Birchmeier, C., Brook, D. & Wigler, M. *Cell* **43**, 615–621 (1985).
- Murray, A. & Kirschner, M. *Science* **246**, 614–621 (1989).
- Lohka, M. J., Hayes, M. K. & Maller, J. L. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3009–3013 (1988).
- Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. *Cell* **54**, 433–439 (1988).
- Dunphy, W. G., Brizuela, L., Beach, D. & Newport, J. *Cell* **54**, 423–431 (1988).
- Labbe, J. C., Lee, M. G., Nurse, P., Picard, A. & Doree, M. *Nature* **335**, 251–254 (1988).
- Labbe, J. C. *et al. Cell* **57**, 253–263 (1989).
- Evans, T., Rosenthal, E., Youngbloom, J., Distel, D. & Hunt, T. *Cell* **33**, 389–396 (1983).
- Hunt, T. *Curr. Op. Cell Biol.* **1**, 268–274 (1989).
- Swenson, K. I., Farrell, K. M. & Ruderman, J. V. *Cell* **47**, 861–870 (1986).
- Murray, A. W. & Kirschner, M. W. *Nature* **339**, 275–280 (1989).
- Minshull, J., Blow, J. & Hunt, T. *Cell* **56**, 947–956 (1989).
- Draetta, G., Luca, F., Westendorp, J., Brizuela, L., Ruderman, J. & Beach, D. *Cell* **56**, 829–838 (1989).
- Labbe, J.-C. *et al. EMBO J.* **8**, 3052–3058 (1989).
- Booher, R. & Beach, D. *EMBO J.* **7**, 2321–2327 (1988).
- Solomon, M., Booher, R., Kirschner, M. & Beach, D. *Cell* **54**, 738 (1988).
- Lee, M. G. & Nurse, P. *Nature* **327**, 31–35 (1987).
- Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. *Cell* **50**, 319–325 (1987).
- Riabowol, K., Draetta, G., Brizuela, L., Vandre, D. & Beach, D. *Cell* **57**, 393–401 (1989).
- Pines, J. & Hunter, T. *Cell* **58**, 833–846 (1989).
- Langan, T. *Methods Cell Biol.* **19**, 127–142 (1978).
- Langan, T. A. *et al. Molec. cell. Biol.* **9**, 3860–3868 (1989).
- Bradbury, E. M., Inglis, R. J. & Matthews, H. R. *Nature* **247**, 257–261 (1974).
- Bradbury, E., Inglis, R., Matthews, H. & Langan, T. *Nature* **249**, 553–556 (1974).
- Gautier, J., Matsukawa, T., Nurse, P. & Maller, J. *Nature* **339**, 626–629 (1989).
- Draetta, G. & Beach, D. *Cell* **54**, 17–26 (1988).
- Inglis, R., Langan, T., Matthews, H., Hardie, D. & Bradbury, E. *Exp. Cell Res.* **97**, 418–425 (1976).
- Nurse, P. *Biochem. Soc. Trans.* 569th Meeting, Sussex, 1191–1193 (1977).
- Morla, A. O., Draetta, G., Beach, D. & Wang, J. Y. L. *Cell* **58**, 193–203 (1989).
- Gould, K. L. & Nurse, P. *Nature* **342**, 39–45 (1989).
- Draetta, G. *et al. Nature* **336**, 738–743 (1988).
- Dunphy, W. G. & Newport, J. W. *Cell* **58**, 181–191 (1989).
- Moreno, S., Nurse, P. & Russell, R. *Nature* **344**, 549–552 (1990).
- Felix, M., Pines, J., Hunt, T. & Karsenti, E. *EMBO J.* **8**, 3059–3069 (1989).
- Cyert, M. & Kirschner, M. *Cell* **53**, 185–195 (1988).
- Murray, A. W., Solomon, M. J. H. & Kirschner, M. W. *Nature* **339**, 280–286 (1989).
- Lehner, C. F. & O'Farrell, P. H. *Cell* **56**, 957–968 (1989).
- O'Farrell, P., Edgar, B., Lakich, D. & Lehner, C. *Science* **246**, 635–640 (1989).
- Ford, C. C. *J. Embryol. exp. Morph.* **89** Suppl., 271–284 (1985).
- Reed, S., Hadwiger, J. & Lorincz, A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4055–4059 (1985).
- Shenoy, S. *et al. Cell* **57**, 763–774 (1989).
- Morgan, D. O., Kaplan, J. M., Bishop, J. M. & Varmus, H. E. *Cell* **57**, 775–786 (1989).
- Cisek, L. & Corden, J. *Nature* **339**, 679–684 (1989).
- McVey, D., Brizuela, L., Mohr, I., Mashak, D. R., Gluzman, Y. & Beach, D. *Nature* **341**, 503–507 (1989).

81. Belle, R., Derancourt, J., Poulhe, R., Capony, J. Ozon, R. & Mulner-Lorillon, D. *FEBS Lett.* **255**, 101-104 (1989).
82. Suzuki, M. *J. molec. Biol.* **207**, 61-84 (1989).
83. Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42-52 (1988).
84. Gerace, L. & Burke, B. A. *Rev. Cell. Biol.* **4**, 335-374 (1988).
85. Bailly, E., Doree, M., Nurse, P. & Bornens, M. *EMBO J.* **8**, 3985-3995 (1989).
86. Akhurst, R. J., Flavin, N. B., Worden, J. & Lee, M. G. *Differentiation* **40**, 36-41 (1989).
87. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. & Yanagida, M. *Cell* **57**, 997-1007 (1989).
88. Boher, R. & Beach, D. *Cell* **57**, 1009-1016 (1989).
89. Doonan, J. H. & Morris, N. R. *Cell* **57**, 987-996 (1989).
90. Norbury, C. & Nurse, P. *Biochim. biophys. Acta* **989**, 85-95 (1989).
91. Russell, P., Moreno, S. & Reed, S. I. *Cell* **57**, 295-303 (1989).
92. Edgar, B. A. & O'Farrell, P. H. *Cell* **57**, 177-187 (1989).
93. Enoch, T. & Nurse, P. *Cell* **60**, 665-673 (1990).
94. Weinert, T. A. & Hartwell, L. H. *Science* **241**, 317-322 (1988).
95. Osmani, S., Engle, D., Doonan, J. & Morris, N. *Cell* **52**, 241-251 (1988).
96. Osmani, S., Pu, R. & Morris, N. *Cell* **53**, 237-244 (1988).
97. Nishimoto, T., Eilen, E. & Basilico, C. *Cell* **15**, 475-483 (1978).
98. Sluder, G. & Lewis, K. J. *exp. Zool.* **244**, 89-100 (1987).
99. Raff, J. & Glover, D. *J. Cell Biol.* **107**, 2009-2019 (1988).
100. Tyson, J. & Kauffman, S. *J. math. Biol.* **1**, 289-310 (1975).
101. Tyson, J. J. *J. theor. Biol.* **126**, 381-391 (1987).
102. Nurse, P. in *DNA and Evolution: Natural Selection and Genome Size* (ed. Cavalier-Smith, T.) 185-196 (Wiley, Chichester, 1985).
103. Kacser, H. & Burns, J. *Symp. Soc. exp. Biol.* **27**, 65-107 (1973).
104. John, P., Sek, F. & Lee, M. *The Plant Cell* **1**, 1185-1193 (1989).

ACKNOWLEDGEMENTS. This article is dedicated to Murdoch Mitchison whose enthusiasm and encouragement first stimulated my interest in the cell cycle. I thank Peter Fantès, Tim Hunt, Andrew Murray and members of the ICRF Cell Cycle Group for their advice during preparation of this manuscript, Audrey Richards and Kevin Crawford for their typing and drawing, and acknowledge the ICRF and MRC for financial support.

ARTICLES

Model calculations of the relative effects of CFCs and their replacements on stratospheric ozone

Donald A. Fisher*, Charles H. Hales*, David L. Filkin*, Malcolm K. W. Ko†, N. Dak Sze†, Peter S. Connell‡, Donald J. Wuebbles‡, Ivar S. A. Isaksen§ & Frode Stordal§

* E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, Delaware 19880-0320, USA

† Atmospheric and Environmental Research Inc., 840 Memorial Drive, Cambridge, Massachusetts 02139, USA

‡ University of California, Lawrence Livermore National Laboratories, PO Box 808, Livermore, California 94550, USA

§ Institute of Geophysics, 1022, 0315 Blinden, Oslo 3, Norway

Because chlorine has been linked to the destruction of stratospheric ozone, the use of many fully halogenated compounds, such as the chlorofluorocarbons CFC-11 and -12, is restricted by international agreement. Hydrohalocarbons are under intensive development as replacements for CFCs. Because they contain hydrogen, these gases are susceptible to tropospheric destruction which significantly shortens their atmospheric lifetimes. Model calculations show that chlorine-containing hydrohalocarbons have less effect on ozone, by an order of magnitude, than their regulated counterparts.

CONCERN over the global environmental consequences of fully halogenated chlorofluorocarbons (CFCs) has sparked development of replacement compounds. The candidates under intensive development are composed of either carbon, hydrogen and fluorine (hydrofluorocarbons or HFCs) or carbon, hydrogen, chlorine and fluorine (hydrochlorofluorocarbons or HCFCs). For simplicity, both classes of compounds are referred to as hydrohalocarbons.

Hydrohalocarbons are destroyed by reaction with atmospheric hydroxyl radicals whereas CFCs are not. Thus, the principal destruction of these compounds occurs in the troposphere rather than the stratosphere. This destruction mechanism significantly shortens their atmospheric lifetimes¹ relative to the lifetimes of CFCs as well as reducing their potential to affect stratospheric ozone. We examine the calculated effects of several one- and two-carbon halocarbons on stratospheric ozone. Estimations of the effects of these halocarbons on global warming are reported separately².

Background

Computer models have long been used to simulate the chemistry of halocarbons and stratospheric ozone. Each halocarbon enters the atmosphere at ground level and, in general, is removed by a combination of chemical processes: reaction with hydroxyl (OH) in the troposphere and the stratosphere, reaction with excited-state oxygen in the stratosphere, and photolytic breakdown by ultraviolet light in the stratosphere. Laboratory measurements indicate that CFCs (for example CFC-11, -12, -113, -114 and -115) are primarily destroyed by ultraviolet light in the stratosphere and, to a lesser degree, by reaction with excited atomic oxygen; reaction with hydroxyl radical is inconsequential. Hydrohalocarbons are removed by all three processes, but the predominant mechanism is reaction with OH in the troposphere. The reaction of the hydrohalocarbons with hydroxyl radicals leads to appreciably shorter atmospheric lifetimes and hence a reduced effect on stratospheric ozone compared with CFCs. Additionally, the HFCs have no calculated effect on ozone because they contain no chlorine and theory indicates that fluorine released in their destruction will rapidly be converted to hydrogen fluoride, which has no effect on ozone.

Early reports were made for the relative effects in terms of an efficiency factor for different chemicals³⁻⁵. The concept of a relative ozone depletion potential (ODP) was introduced in 1981 (ref. 6) and was adopted as a quick reference for estimating the relative potential of these trace gases to destroy stratospheric ozone. Several papers have reported model results as ODPs⁷⁻⁹. International regulation of fully halogenated CFCs under the Montreal Protocol is based on the concept of ODP in order to establish relative weighting factors for each chemical.

ODP values for sixteen gases have been calculated by four atmospheric modelling groups; Atmospheric and Environmental Research Inc. (AER), Du Pont Central Research (Du Pont), Lawrence Livermore National Laboratory (LLNL), and the University of Oslo. Here both one- and two-dimensional

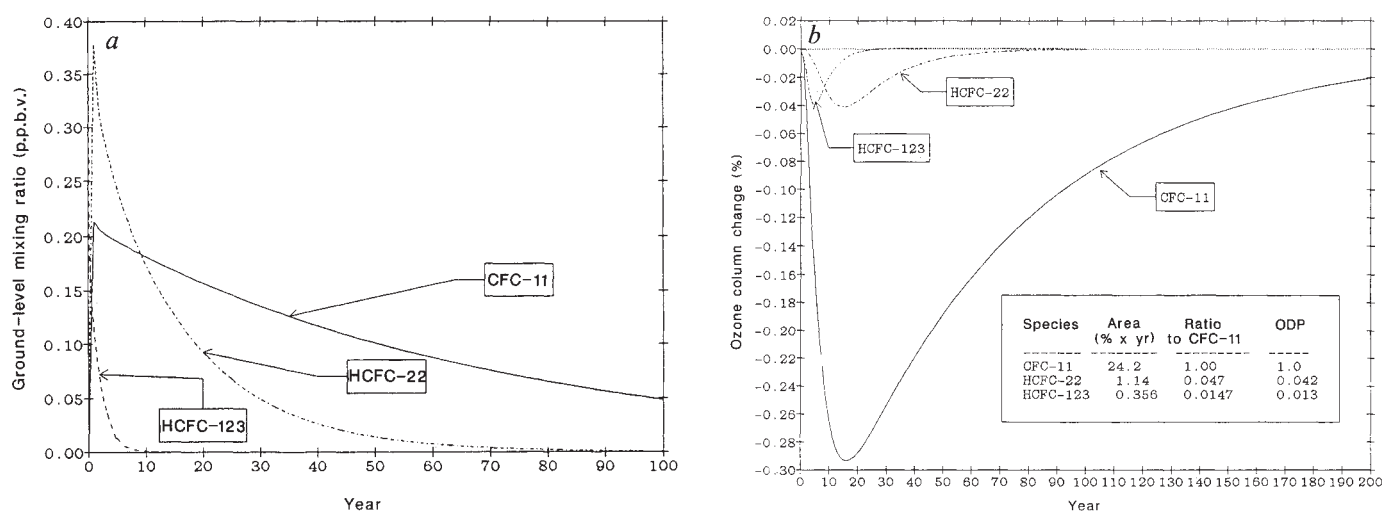


FIG. 1 Calculated responses following a single release of fluorocarbon gases (scaled to 5.0×10^9 kg release) for: a, tropospheric mixing ratio of

each gas, and b, column ozone change.

models were used^{7,9-17}. The halocarbons considered and their chemical formulae are listed in Table 1.

Definition basis

Ozone depletion potential is traditionally defined as a steady-state ratio of calculated change in the ozone column from a constant emission of gas into the atmosphere relative to the calculated depletion for the same (mass) emission of the reference gas CFC-11. Column ozone is the total amount of ozone between the Earth's surface and space, mathematically computed from atmospheric models as the vertical integral of ozone concentration (molecules cm^{-3}) through the entire atmosphere. ODP provides a measure of the cumulative effect on ozone for each unit released into the atmosphere. It yields a single value for each compound rather than a time-varying multitude of

values and it provides an estimate of the maximum calculated effect of a compound compared to the maximum calculated effect of CFC-11 on an equal-mass basis.

The first, and most critical, reason for choosing this definition is that it also represents the cumulative effect on atmospheric ozone of each unit mass of gas released. This is shown by the following test performed using the Du Pont one-dimensional model to determine the relative effects of single releases of HCFC-123, HCFC-22 and CFC-11. The intent is to demonstrate the relative effects of unit mass emissions on stratospheric ozone. To represent this in the model, the emission level was scaled to a pulse of 5×10^9 kg for each halocarbon. Subsequent model years had zero emissions.

Transient calculated results are shown in Fig. 1. Figure 1a shows the calculated tropospheric concentration of each gas,

TABLE 1 Chemical rate data used in ODP calculations

Species	Chemical formula	Ultraviolet photolysis cross-section reference	OH reactions			$\text{O}^{(1\text{D})}$ reactions		
			Reaction A	Rate E/R	Reference	Reaction rate constant†	Branch ratio‡	Reference§
CFC-11	CCl_3F	22	—	—	—	2.3 (–10)	0.75	22
CFC-12	CCl_2F_2	22	—	—	—	1.4 (–10)	0.86	22
CFC-113	$\text{CCl}_2\text{FCClF}_2$	24	—	—	—	2.0 (–10)	0.80	23
CFC-114	$\text{CClF}_2\text{CClF}_2$	24	—	—	—	1.6 (–10)	0.80	23
CFC-115	CClF_2CF_3	24	—	—	—	0.89 (–10)	0.80	23
HCFC-22	CHClF_2	22	1.2 (–12)	1,650	20	1.0 (–10)	1.0	20
HCFC-123	CF_3CHCl_2	21	6.4 (–13)	850	20	2.3 (–10)	1.0	20
HCFC-124	CF_3CHClF	21	6.6 (–13)	1,250	20	1.0 (–10)	1.0	H
HFC-125	CF_3CHF_2	—	3.8 (–13)	1,500	20	0.5 (–10)	1.0	20
HFC-134a	$\text{CF}_3\text{CH}_2\text{F}$	A	1.7 (–12)	1,750	20	0.5 (–10)	1.0	H
HCFC-141b	CCl_2FCH_3	21	2.7 (–13)	1,050	20	1.5 (–10)	1.0	H
HCFC-142b	CClF_2CH_3	21	9.6 (–13)	1,650	20	1.4 (–10)	1.0	20
HFC-143a	CF_3CH_3	—	2.6 (–13)	1,500	20	0.6 (–10)	1.0	20
HFC-152a	CHF_2CH_3	A	1.5 (–12)	1,100	20	1.0 (–10)	1.0	H
Carbon tetrachloride	CCl_4	22	—	—	—	3.3 (–10)	0.86	22
Methylchloroform	CCl_3CH_3	22	5.0 (–12)	1,800	22	3.18 (–10)	0.80	23

* Reaction rate constant $k = A \exp [-E/(RT)]$, where A and k have the units of $\text{cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ (base 10 exponent in parentheses).

† Reaction rate constant with units $\text{cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ independent of temperature.

‡ Fraction of $\text{O}^{(1\text{D})}$ disappearances proceeding through reaction channel, remainder pass through quenching channel to $\text{O}^{(3\text{P})}$ and no reaction with the halocarbon.

§ Measurements reported in ref. 23 were used. Data for remaining gases generated using the estimation formula developed in that reference. Branching ratios of 0.8 were assumed values.

Reference key: A, Magid (personal communication); H, Hampson (personal communication).

peaking at the release year and then diminishing over time as a result of chemical removal in the atmosphere. The decay time constant (on an exponential scale) equals the calculated lifetime of the gas.

Figure 1b shows the change in column ozone for all cases. Differences are apparent for each chemical because of the different effect they each have on stratospheric chlorine chemistry. First, the wt% of chlorine in each species is substantially different, ranging from 71% in CFC-11 to 41% in HCFC-22. Second, location of decomposition is important; HCFCs-123 and -22 are mainly destroyed in the troposphere such that only a fraction of their chlorine reaches the stratosphere, whereas CFC-11 passes through the troposphere intact, such that all the chlorine is transported to the stratosphere where it eventually is broken down (primarily through photolysis) to inorganic chlorine.

The inset table shows the cumulative (time-integrated) depletion from emission of each halocarbon; they are closely proportional to the ODP values derived from the steady-state calculations. The mathematical equivalence between ODP and this transient problem has been shown³⁰. In summary, this exercise validates ODP as a measure of the cumulative effect on stratospheric ozone for each mass unit emitted to the atmosphere.

We should, however, recognize the limitations of a single-valued definition of ODP. For instance, an ODP defined at steady state is not representative of relative transient effects, especially during the early years of emissions. This is illustrated in Fig. 2 where the time-evolution of tropospheric concentration of two compounds following the onset of a sustained constant emission level for each is shown. One of the compounds has a lifetime of 100 years (comparable to the lifetimes of CFCs) and the other compound has a lifetime of 5 years (comparable to the lifetimes of hydrohalocarbons). We see that the ratio of the relative atmospheric concentrations does not approach steady state until about 400 years after emission. As each compound's effect on stratospheric ozone at any time is (to first order) proportional to its atmospheric abundance, the transient relative effect has a similar time pattern. Thus, early on, the relative effect is higher than the (steady-state) ODP value because the steady-state impact on ozone from the short-lived species has been realized in ten years whereas the effect from the long-lived species is still well below its steady-state value.

Definitions

To make the ODP definition consistent among the models, the following criteria were selected. (1) Depletion level: the calculations

are based on emission rates of each compound required to give a modelled steady-state ozone depletion of ~1%. This value was selected to yield results large enough to avoid the noise level inherent with numerical models, yet small enough to remain in the linear perturbation region. (2) Trace-gas levels: changing concentrations of other trace gases affect calculated tropospheric OH levels^{18,19} and future depletions. However, we chose to base the calculations on current levels of CO₂, CH₄, CO and N₂O because of the uncertainties in future concentrations. The constant concentration assumption was chosen for clarity and simplicity. (3) Chlorine levels: as the long-lived CFCs are present in today's atmosphere and will affect chlorine chemistry over the timescales for which hydrohalocarbons may be used, background halocarbon concentrations were assumed constant at current levels (3.0 p.p.b.v. (parts per 10⁹ by volume)) in the stratosphere with the exception of the Oslo model which used a background amount of 5.2 p.p.b.v. Previous calculations with the Oslo model indicate little effect on the derived ODPs from the assumed chlorine background. (4) Bromine chemistry: current levels of bromine compounds were included by some models. Subsequent calculations have shown that assumed bromine levels do not affect ODP values to any significant degree.

Using the above criteria, the ODPs were calculated as:

$$\text{ODP} = \frac{\left(\frac{\text{Calculated O}_3 \text{ depletion due to compound X}}{\text{Steady state emission rate of compound X to give depletion of } \sim 1\%} \right)}{\left(\frac{\text{Calculated O}_3 \text{ depletion due to CFC-11}}{\text{Steady-state emission rate of CFC-11 to give depletion of } \sim 1\%} \right)}$$

CFC-11 is used as the reference gas consistent with previous work⁶.

Model calculations

Atmospheric chemistry models use mathematical expressions to describe the chemical reaction rates, transport and photolysis processes that determine the chemical fate of the halocarbons.

Reaction rate constants and photolysis cross-sections required as model input were obtained from a number of sources. When available, rate data recently re-evaluated by Hampson *et al.*²⁰, R. F. Hampson (personal communication) and Molina²¹ have been used. The secondary data source was the most recent evaluation of rate data by NASA²². In the absence of these recommendations, some reaction rate parameters were obtained from the open literature^{23,24}. Some data were only available from unpublished sources (H. Magid, personal communication). Finally, no photolysis measurements could be found for HFC-125 and HFC-143a, so destruction by photolysis was assumed

TABLE 2 Model-calculated lifetimes in years

Species	1-D model results			2-D model results			
	LLNL	AER	Du Pont	Oslo	LLNL	AER	Du Pont
CFC-11	80	60	71	60	52	47	46
CFC-12	154	125	154	195	101	95	118
CFC-113	96	96	117	101	79		
CFC-114	209	260	319	236	197		
CFC-115	680	690	548	522	393	399	
HCFC-22	20	20.0	16	17	15	24	12.7
HCFC-123	1.9	2.1	1.6	1.7	1.5	2.4	1.2
HCFC-124	8.4	8.8	6.9	7.4	6.5	10	5.3
HFC-125	37	37	25		27	43	19
HFC-134a	21	21	16		15	24	12.5
HCFC-141b	8.9	9.4	7.8	8.0	6.9	11	5.8
HCFC-142b	25	25	19	21.0	19	28	15.1
HFC-143a	54	52	42		40		
HFC-152a	2.1	2.3	1.7		1.5	2.7	1.3
CCl ₄	73	53	61	52.2	47	40	40
CH ₃ CCl ₃	7.4	7.4	6.0	6.3	5.8	7.8	4.7

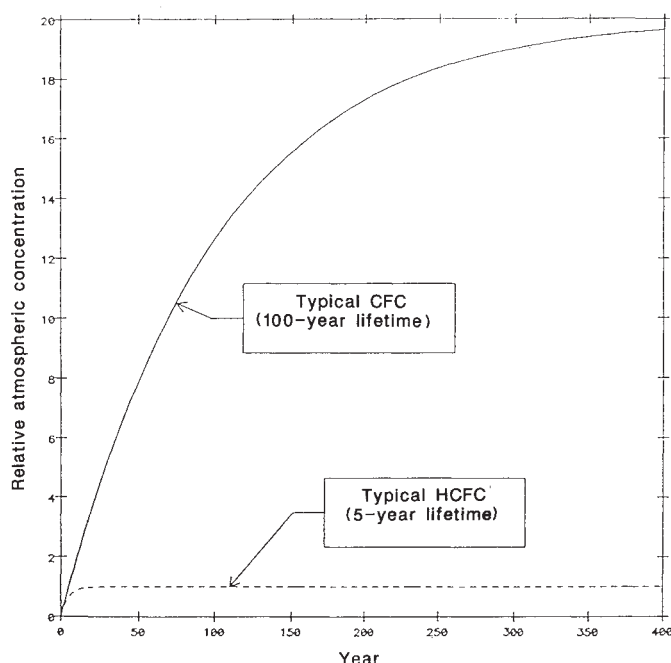


FIG. 2 Transient atmospheric concentrations of halocarbons with 5- and 100-year lifetimes at constant (molecular) emissions.

to be zero. Table 1 lists reference sources, and where possible, the values for each of the kinetic parameters. For these calculations, all chlorine from each halocarbon was assumed to be released at the point of halocarbon destruction. The most recent recommendations from NASA²² were used to represent chemical reactions involving inorganic chlorine. All models use their 'best' representations for transport and radiation processes, generally derived from first principles and consistent with observed values for key trace species.

Both one- and two-dimensional models have been used for these evaluations. One-dimensional models calculate the average altitudinal variation of the relevant atmospheric chemical processes. These models are easy to operate and evaluate. Two-dimensional models, on the other hand, allow effects over all latitudes and seasons to be examined. As such, their added complexity is offset by better representation of nonlinear

characteristics of global stratospheric transport and radiative processes. The ODP values from the two-dimensional models are calculated from changes in global (averaged with respect to both latitude and seasons) amount of ozone.

The calculated atmospheric lifetime of each gas species provides a key comparison for modelled results. Table 2 shows a comparison of lifetime values calculated by the models. ODP values are reported in Table 3. Note that the HFCs (molecules without chlorine) have zero effect on ozone, and therefore ODP=0. Each value of the ODP is reported to 2 figures—in reality, the results are credible to less than two significant figures because of uncertainties in model input parameters.

Differences in calculated tropospheric hydroxyl concentrations account for many of the differences in the calculated hydrohalocarbon lifetimes. Several factors may contribute to the differences in the calculated concentrations of tropospheric OH. First, there is inadequate information on the distributions of a number of gases (for example, CO, O₃ and NO_x) that have critical roles in the determination of tropospheric OH levels. Second, there are uncertainties in many tropospheric processes, especially in rates for key reactions.

Analysis of data for atmospheric CH₃CCl₃ may provide a reasonable although indirect check on the calculated lifetime of CH₃CCl₃ and therefore the global average concentration of OH. Based on the observed trends of CH₃CCl₃ and the estimated emission data, analysis indicates a globally averaged CH₃CCl₃ atmospheric lifetime of 6.3 ± 1 years^{25,26} compared with model-calculated values ranging from 4.7 to 7.8 years (see Table 2) from the different models. The deduced average OH from CH₃CCl₃ data, although useful for placing important constraints on theoretical models, must be interpreted as an appropriate density and temperature-weighted average characteristic of CH₃CCl₃. Because the concentration of OH is expected to fluctuate appreciably in both time and space, using globally averaged OH deduced from CH₃CCl₃ to calculate lifetimes for other HCFCs may not necessarily be accurate, particularly for those HCFCs with lifetimes substantially different from that of CH₃CCl₃. The approach used here is to calculate tropospheric OH based on chemical models rather than adopt a single OH value derived from CH₃CCl₃ data.

To account for differences in ODPs due to differences in model-calculated OH, the calculated ODPs from each model can be scaled using the ratio of measured lifetime for CH₃CCl₃ of 6.3 years^{25,26} to the calculated CH₃CCl₃ lifetimes from the corresponding model. This normalization leads to a more consistent set of ODP values as shown in Table 4. These calculations indicate that the HCFCs have ozone-depletion potentials

TABLE 3 Ozone Depletion Potential

Species	1-D model results			2-D model results			
	LLNL	AER	Du Pont	Oslo	LLNL	AER	Du Pont
CFC-11	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CFC-12	1.0	0.92	1.0	0.92	0.87	0.88	0.89
CFC-113	0.82	0.83	0.89	0.86	0.76		
CFC-114	0.76	0.63	0.79	0.82	0.56		
CFC-115	0.43	0.36	0.45	0.40	0.27	0.37	
HCFC-22	0.053	0.057	0.042	0.046	0.043	0.071	0.032
HCFC-123	0.016	0.019	0.013	0.013	0.016	0.027	0.013
HCFC-124	0.019	0.021	0.016	0.018	0.017	0.030	0.013
HFC-125	0	0	0	0	0	0	0
HFC-134a	0	0	0	0	0	0	0
HCFC-141b	0.081	0.092	0.066	0.089	0.081	0.14	0.065
HCFC-142b	0.059	0.056	0.053	0.056	0.045	0.077	0.035
HFC-143a	0	0	0	0	0	0	0
HFC-152a	0	0	0	0	0	0	0
CCl ₄	1.1	1.16	1.1	1.2	1.1	0.95	1.2
CH ₃ CCl ₃	0.11	0.14	0.092	0.14	0.12	0.20	0.11

TABLE 4 HCFCs ODPs scaled by methylchloroform lifetime of 6.3 years

Species	1-D model results			2-D model results			
	LLNL	AER	Du Pont	Oslo	LLNL	AER	Du Pont
HCFC-22	0.045	0.049	0.044	0.046	0.047	0.057	0.043
HCFC-123	0.013	0.016	0.013	0.013	0.017	0.022	0.017
HCFC-124	0.016	0.018	0.017	0.018	0.019	0.024	0.017
HCFC-141b	0.069	0.078	0.069	0.089	0.089	0.11	0.088
HCFC-142b	0.051	0.048	0.055	0.056	0.050	0.062	0.047
CH ₃ CCl ₃	0.094	0.12	0.096	0.14	0.13	0.16	0.149

one-tenth or less of CFC-11 and that the values calculated by different modelling groups agree fairly well.

Uncertainties

None of the above calculations of the effect on ozone considers the potential effects of heterogeneous chemistry in the lower stratosphere, particularly within the vortex occurring at either pole during late winter and early spring. Available observations indicate that the observed Antarctic ozone destruction is occurring as a result of heterogeneous reactions associated with polar stratospheric clouds resulting in efficient chlorine-catalysed ozone destruction²⁷. Under these conditions, chlorine-induced ozone loss in the late winter/early spring polar stratosphere is related to the total amount of available chlorine at the height of the polar stratospheric clouds, that is, below ~25 km. At present, the inclusion of such effects is premature, as modelling of heterogeneous chemistry in general, and the polar phenomena in particular, is still in the early stages. Furthermore, increases in carbon dioxide and other greenhouse gases may result in a cooling of the stratosphere, thereby causing an increase in the occurrence of polar stratospheric clouds.

Although the inclusion of polar chemical effects would increase the magnitude of calculated global ozone depletion from each species, the impact on the (relative) ODP value is less clear. The overall impact of these phenomena on the magnitude of the global ODP depends on the impact of polar processes on ozone at lower latitudes either by dilution effects²⁸ or by a 'chemical processor' mechanism²⁹, and the inter-species

differences in amounts of reactive chlorine delivered to the polar regions. Until these processes are better understood and incorporated in models, we can supply only rough estimates of the relative contribution of each compound by the amount of chlorine that could be available for affecting polar processes. Estimates of relative contributions to stratospheric chlorine are available³⁰.

Although the calculated values of ODPs agree reasonably well from these models, uncertainties in the values still exist because of uncertainties in modelled chemistry and dynamics. Because the reaction with OH dominates the chemistry of the HCFCs, uncertainties in the model-calculated OH values remain a chief source of uncertainty for both the lifetimes and ODPs of these compounds. Uncertainties for some compounds may be reduced with new laboratory data on the ultraviolet and infrared absorption properties and the rate constants with hydroxyl for the HCFCs and HFCs.

Applications

ODPs of the hydrohalocarbons are generally one-tenth or less than those of CFC-11 and CFC-12. The reduction in ODP that might be expected to come from replacement of a CFC by a hydrohalocarbon can be estimated by taking the ratio of the ODP of the hydrohalocarbon to the ODP of the CFC it might replace. For example, the reduction in ODP in replacing CFC-12 by HCFC-22 is a factor of 19 (0.93/0.049). Of course, the relative quantities of the compound required as replacement must also be taken into account. □

Received 30 August 1989; accepted 23 January 1990.

- Makide, Y. & Rowland, F. S. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5933-5937 (1981).
- Fisher, D. A., Hales, C. H., Wang, W.-C., Ko, M. K. W. & Sze, N. D. *Nature* **344**, 513-516 (1990).
- Wofsy, S. C. & McElroy, M. B. *Can. J. Chem.* **52**, 1582-1591 (1974).
- Donahue, T. M., Cicerone, R. J., Liu, S. C. & Chameides, W. L. *Geophys. Res. Lett.* **3**, 105-108 (1976).
- Robins, D. E. & Stolarski, R. S. *Geophys. Res. Lett.* **3**, 603-606 (1976).
- Wuebbles, D. J. *Rep. UCID-18924* (Lawrence Livermore National Laboratory, Livermore, 1981).
- Wuebbles, D. J. *J. geophys. Res.* **88**, 1433-1443 (1983).
- Hammit, J. K. *et al.*, *Nature* **330**, 711-716 (1987).
- Rognrud, B., Isaksen, I. S. A. & Strodel, F. in *Ozone in the Atmosphere* (eds Bojkov, R. D. & Fabian, P.) 609-612 (Deepak Publishing, Hampton, Virginia, 1989).
- Ko, M. K. W., Sze, N. D., Livshits, M., McElroy, M. B. & Pyle, J. A. *J. Atmos. Sci.* **41**, 2381-2408 (1984).
- Ko, M. K. W., Tung, K. K., Weisenstein, D. K. & Sze, N. D. *J. geophys. Res.* **90**, 2313-2329 (1985).
- Kinnison, D., Johnston, H. & Wuebbles, D. J. *J. geophys. Res.* **93**, 14165-14175 (1988).
- Miller, C., Filkin, D. L., Owens, A. J., Steed, J. M. & Jesson, J. P. *J. geophys. Res.* **66**, 12039-12065 (1981).
- Miller, C., Steed, J. M., Filkin, D. L. & Jesson, J. P. *Atmos. Environ.* **15**, 729-942 (1981).
- Stordal, F., Isaksen, I. S. A. & Horntveth, K. *J. geophys. Res.* **90**, 5757-5776 (1985).
- Sze, N. D. & Ko, M. K. W. *Atmos. Environ.* **15**, 1301-1367 (1981).
- Wuebbles, D. J. & Kinnison, D. E. in *Ozone in the Atmosphere* (eds Bojkov, R. D. & Fabian, P.) 605-608 (Deepak Publishing, Hampton, Virginia, 1989).
- Sze, N. D. *Science* **195**, 673-675 (1977).
- Chameides, W. L., Liu, S. C. & Cicerone, R. C. *J. geophys. Res.* **82**, 1795-1798 (1977).

- Hampson, R. F., Kurylo, M. J. & Sander, S. P. in *World Meteorological Organization, Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20*, Vol. II, 47, Appendix: AFEAS Report (Geneva, 1989).
- Molina, M. J. in *World Meteorological Organization, Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20*, Vol. II, 111, Appendix: AFEAS Report (Geneva, 1989).
- DeMore, W. B. *et al.* *JPL Publication* 87-41 (Jet Propulsion Laboratory, Pasadena, 1987).
- Davidson, J. A., Schiff, H. I., Brown, T. J. & Howard, C. J. *J. chem. Phys.* **69**, 4277-4279 (1978).
- Hubrich, C. & Stuhl, F. *J. Photochem.* **12**, 93-107 (1980).
- Prinn, R. *et al.* *Science* **238**, 945-950 (1987).
- Watson, R. T., Prather, M. J. & Kurylo, M. J. *Present state of knowledge of the upper atmosphere 1988: An assessment report* (to congress), NASA Reference Publication 1208 (Washington, 1988).
- Solomons, S. *Rev. Geophys.* **26**, 141-148 (1988).
- Sze, N. D. *et al.* *J. geophys. Res.* **94**, 11521-11528 (1989).
- Tuck, A. F. *J. geophys. Res.* **94**, 11687-11737 (1989).
- Fisher, D. A. *et al.* in *World Meteorological Organization, Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20*, Vol. II, 297, Appendix: AFEAS Report (Geneva, 1989).

ACKNOWLEDGEMENTS. We thank Allied-Signal Corporation, Atochem, E. I. Du Pont de Nemours and Company, Hoechst Aktiengesellschaft, ICI Chemicals and Polymers Ltd, and Montefluos SPA. Work at AER, Inc. is also supported by NASA, Upper Atmospheric Theory and Data Program. Work at LLNL is also supported by NASA as well as the US Department of Energy.

Model calculations of the relative effects of CFCs and their replacements on global warming

Donald A. Fisher*, Charles H. Hales*, Wei-Chyung Wang†, Malcolm K. W. Ko† & N. Dak Sze†

* E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, Delaware 19880-0320, USA

† Atmospheric and Environmental Research Inc., 840 Memorial Drive, Cambridge, Massachusetts 02139, USA

Halocarbons can contribute to global warming by absorbing long-wave radiation. Concern over the depletion of stratospheric ozone has led to international agreements that restrict future uses of fully halogenated compounds, such as chlorofluorocarbons CFC-11 and -12. But most compounds proposed as replacements also absorb long-wave radiation, and so their potential contributions as greenhouse gases need to be assessed. Model calculations show that the replacement compounds have an effect about an order of magnitude less than that of their regulated counterparts.

THE international conventions (Montreal 1987) regulating the future use of chlorofluorocarbons (CFCs) stem from the recognition that these compounds may affect stratospheric ozone. Another environmental concern involving these compounds is their role as greenhouse gases. Two inherent chemical characteristics enhance their effectiveness as greenhouse gases. First, they absorb radiation in the 8–12- μm region of the spectrum where naturally occurring gases absorb weakly. Therefore changes in the absorption processes in this region critically influence the radiative cooling processes of the Earth. Second, CFCs have very long chemical lifetimes which allow them to build up significantly in the atmosphere and to exert a proportionate impact on the radiative balance of the Earth. But the chemicals proposed as replacements for the CFCs also absorb infrared energy in the same window of the infrared spectrum, so that their effectiveness as greenhouse gases must also be assessed and compared with that of the CFCs. To this end, we have used two atmospheric models (developed separately). Each model simulates the chemical reactions and radiative balance of the atmosphere. We normalize the calculated effects with respect to CFC-11; the results of the two models agree reasonably well if the assumptions about chemical lifetimes are consistent. Our chief conclusion is that the potential of the replacement compounds to affect global warming is significantly less than that of the CFCs, chiefly because their atmospheric lifetimes are shorter.

Background

Candidate replacements are composed of either carbon, hydrogen and fluorine (hydrofluorocarbons or HFCs) or carbon, hydrogen, chlorine and fluorine (hydrochlorofluorocarbons or HCFCs). For simplicity, both classes of compounds are referred to as hydrohalocarbons. Because they contain hydrogen, the hydrohalocarbons are subject to destruction in the atmosphere through reaction with hydroxyl radicals. This destruction mechanism leads to much shorter atmospheric lifetimes for the hydrocarbon compounds (see ref. 1 for a discussion of the chemistry that determines the atmospheric lifetimes of these gases). By contrast, the only known destruction process of CFCs is through photolysis in the upper stratosphere.

We quantify estimates of the greenhouse effect of each com-

pound in terms of a relative potential to enhance global warming—halocarbon global warming potential (HGWP)—which is similar to the ozone depletion potential (ODP) in concept. No attempt is made to calculate HGWPs for non-halocarbon gases such as carbon dioxide or methane. Because of the current atmospheric concentrations and spectral locations of the infrared absorption bands of these other gases, calculated global warming is not a linear function of their atmospheric concentration. By contrast, a calculated warming is linearly proportional to concentrations of halocarbons (over the range of expected commercial levels). Thus, greenhouse warming potentials for carbon dioxide and methane would not be meaningful in this context.

Using two atmospheric, radiative-convective photochemical models, developed at Atmospheric and Environmental Research Inc. (AER) and Du Pont Central Research (Du Pont), respectively, we have calculated HGWP values for sixteen gases. The halocarbons considered are listed in Table 1 and are the same as those studied in ref. 1.

Definition basis

HGWP is defined as the ratio of the steady-state calculated warming for a fixed release of a gas relative to the calculated warming for the same release (by mass) of reference gas CFC-11. This definition was chosen as a representative measure because it provides a measure of the cumulative effect on the radiative balance over its chemical lifetime for each unit released into the atmosphere (see below), and because the HGWP yields a single value for each compound rather than a time-varying multitude of values.

The first reason is perhaps the most important, in that HGWP estimates the cumulative effect on global warming for each unit released. As a test, the Du Pont model was used to quantify the relative effects of the emission of each single mass unit on global warming. To illustrate this point using the model, the emission level was scaled to a single 5×10^9 -kg release for each halocarbon.

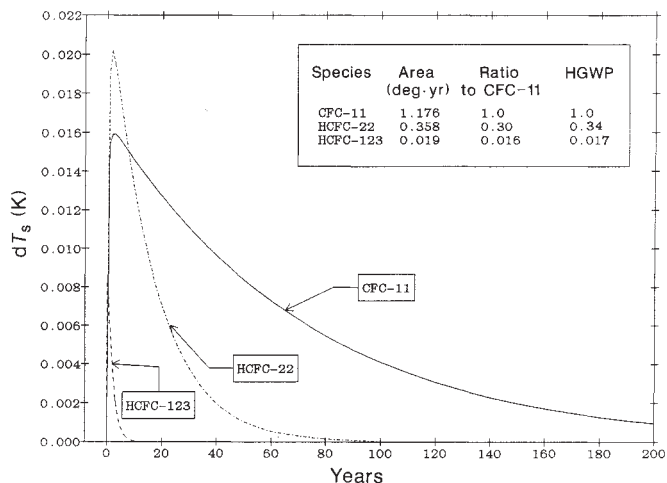


FIG. 1 Calculated change in surface temperature for individual releases of gases (scaled to 5×10^9 kg).

TABLE 1 Total band strengths of halocarbons

Species	Chemical formula	Ref. 7	Ref. 6	Ref. 5	Gehring*	Magid*‡
CFC-11	CCl ₃ F	—	2,389	2,566	—	2,389†
CFC-12	CCl ₂ F ₂	—	3,267	3,364	3,310	3,240†
CFC-113	CCl ₂ FCF ₂	4,822	3,507	—	3,126	3,401†
CFC-114	CClF ₂ CCF ₂	5,935	3,937	—	—	4,141†
CFC-115	CCF ₂ CF ₃	—	—	—	—	4,678†
HCFC-22	CHClF ₂	—	—	—	2,399	2,554†
HCFC-123	CF ₃ CHCl ₂	—	—	—	2,552	2,859†
HCFC-124	CF ₃ CHClF	—	—	—	4,043†	—
HFC-125	CF ₃ CHF ₂	—	—	—	3,908†	—
HFC-134a	CF ₃ CH ₂ F	—	—	—	3,169	3,272†
HCFC-141b	CCl ₂ CF ₂ CH ₃	—	—	—	1,732	1,912†
HCFC-142b	CClF ₂ CH ₃	—	—	—	2,474	2,577†
HFC-143a	CF ₃ CH ₃	—	—	—	3,401†	—
HFC-152a	CHF ₂ CH ₃	—	—	—	—	1,648†
Carbon tetrachloride	CCl ₄	—	—	—	1,195†	—
Methylchloroform	CH ₃ CCl ₃	—	—	—	1,184	1,209†

* Personal communication.

† Infrared data used in model calculations.

‡ The infrared data from Magid (1988) were given with spectra resolutions of 0.5 to 0.25 cm⁻¹. The integrated band strengths are given here so that they can be compared with other data.Units are atm⁻¹ cm⁻² STP.

The transient calculated warmings due to HCFC-123, HCFC-22 and CFC-11 from this test are shown in Fig. 1 for the 200-yr period following release. For each case, the warming peaks very rapidly following release and tails off exponentially (with a time constant equal to the atmospheric lifetime of each species). As seen in the inset, the time-integrated relative warmings for single releases are essentially equal to the relative HGWP values determined from steady-state calculations, thereby verifying that HGWP is a measure of cumulative warming.

Definitions

To make the definition of HGWP consistent between models, the following criteria have been selected. (1) Trace gas levels: changing the concentration of other trace gases will affect the calculated future equilibrium temperature rise from the gases under evaluation for two reasons. First, if there is overlap of absorption spectra, certain absorption bands have less effect. Second, chemistry and therefore lifetime can be affected by perturbation of these chemicals. Current ground-level concentrations of CO₂, CH₄ and N₂O were used in model calculations. (2) Reference gas: CFC-11 has been chosen as the reference compound for HGWP calculations to have a reference material consistent for both HGWP and ODP. (3) Gas perturbation levels: the calculations are based on emission rates that yield modelled responses large enough to be significantly above the 'noise levels' of the numerical models. (4) Specific surface-temperature change: we define the calculated surface temperature increase for a one part per 10⁹ increase in surface mixing ratio of any gas to be the specific surface-temperature change, dT_s.

For any gas, the general definition of HGWP is:

$$\text{HGWP} = \frac{\text{Calculated (steady-state) infrared forcing due to compound X}}{\text{Emission rate of compound X}} \div \frac{\text{Calculated (steady-state) infrared forcing due to CFC-11}}{\text{Emission rate of CFC-11}}$$

Infrared forcing is the net change in infrared flux at the tropopause.

Because the calculated infrared forcing for a given emission is proportional to the product of dT_s and atmospheric abundance, and because atmospheric abundance is proportional to the ratio of (lifetime × emission rate) to molecular weight, an equivalent definition is:

$$\text{HGWP} = \frac{\frac{(dT_s(x))(\text{lifetime}(x))}{\text{molecular weight}(x)}}{\frac{(dT_s(\text{CFC-11}))(\text{lifetime}(\text{CFC-11}))}{\text{molecular weight}(\text{CFC-11})}}$$

Some of the gases have the potential to change atmospheric heating rates indirectly because they can chemically influence the distribution of ozone which affect both solar and long-wave heating rates. An examination of model results indicates that this is a second-order effect⁴.

Model calculations

Steady-state altitudinal concentration profiles determined by the models provide the main input to the radiative calculations. Given these concentration profiles, the effect of each gas is calculated using a radiative-convective model which uses infrared absorption spectra to quantify the ability of a gas to absorb infrared energy and thereby influence the Earth's heat balance. Equilibrium temperature profiles are calculated from an energy balance such that solar heating is in equilibrium with infrared cooling at all altitudes throughout the stratosphere. In the troposphere, a constant lapse rate is assumed to calculate the convective heating which balances the solar heating and infrared cooling. The reference atmosphere is model-derived, based on current ground-level values for trace gases. The calculation accounts for the amount of energy absorbed by each infrared gas (the band strength) at specified wavelengths (the band location) including spectral overlap with other gases. Quantitative infrared data for this input are available from the literature for CFCs (refs 5–7). Measurements for the HCFCs and the HFCs were obtained from industry laboratories (H. Magid; and D. G. Gehring, personal communications).

Total band strengths available for these calculations, as shown in Table 1, agree within ~10% with the exception of those of Rogers and Stephens⁷. The band strengths used for the model calculations are marked with a dagger (†). As there seem to be systematic differences between laboratories for band-strength measurements, the values from a common database (H. Magid, personal communication) were used because it covered most of the compounds of interest in this study. For compounds not available from this source, data from D. G. Gehring (personal communication) were used. The data sets were determined at room temperature and in the absence of temperature-dependent data sets, band strengths were assumed to be constant throughout the atmosphere.

Individual band strengths and band locations are important in these calculations. Table 2 details the locations and strengths for the absorption spectra used here, broken down into the 'bin' structure of the Du Pont model. The greatest amount of absorption for many species occurs between 1,070 and 1,400 cm⁻¹. Absorption by CH₄ and N₂O in the background atmosphere occurs in this region of the spectrum. Some species (for example,

TABLE 2 Absorbance bands used

Bin limit (cm ⁻¹)																	
Min	Max	CFC-11	CFC-12	CFC-113	CFC-114	CFC-115	HCFC-22	HCFC-123	HCFC-124	HFC-125	HFC-134a	HCFC-141b	HCFC-142b	HFC-143a	HFC-152a	CCl ₄	CH ₃ CCl ₃
440.0	500.0	0	0	0	0	0	0	0	24.0	0	0	0	0	0	0	0	0
500.0	555.0	0	0	0	0	0	0	0	49.6	30.6	0	0	0	0	0	0	0
555.0	616.256	0	0	0	23.9	0	0	0	21.3	61.0	0	82.2	0	65.1	0	0	0
616.256	618.030	0	0	0	3.8	0	0	0	0	0.4	0	0	0	3.4	0	0.8	0
618.030	647.085	0	0	14.7	17.1	20.9	0	0	0	6.2	10.8	0	0	18.6	0	9.3	0
647.085	648.825	0	0	1.2	0	8.1	0	0	0	0.3	2.0	0	0	0	0	0.3	0
648.825	667.370	0	19.8	19.1	0	21.8	0	27.6	11.2	2.5	64.9	0	17.2	0	0	0	0
667.370	669.250	0	3.0	0	3.0	1.3	0	6.9	0.6	0	9.2	0	6.6	0	0	0	0
669.250	718.550	0	23.3	0	14.2	0	0	77.5	137.4	24.5	52.2	0	160.6	0	0	0	172.1
718.550	720.800	0	0	0	0	0	0	0	0	11.4	0	0	0	0	0	0	73.2
720.800	775.0	0	0	0	0	107.4	0	43.8	0	79.0	0	542.1	0	0	0	260.0	448.5
775.0	862.0	1,675.0	21.8	722.4	578.6	0	566.3	449.6	200.5	46.8	69.6	33.3	0	16.5	17.1	910.0	24.8
862.0	924.0	32.0	962.0	706.1	394.0	0	0	83.8	294.9	120.3	14.4	97.7	406.2	13.2	65.6	11.5	0
924.0	928.0	7.4	144.5	32.3	66.7	0	0	0	0	0.7	0	45.2	2.9	1.4	16.0	1.1	0
928.0	934.0	15.0	228.0	30.7	64.2	0	0	0	0	1.1	0	54.3	0	2.4	28.2	1.7	0
934.0	1,004.0	34.6	67.2	36.9	57.4	923.3	0	0	0	0	220.8	70.3	203.0	423.4	178.7	0	0
1,004.0	1,036.0	0	0	71.2	19.3	51.7	0	0	0	0	11.8	9.0	5.7	10.4	7.6	0	9.4
1,036.0	1,042.0	1.9	0	90.7	53.2	0	0	0	0	0	2.5	0	0	0	1.2	0	2.9
1,042.0	1,048.0	2.8	0	105.6	129.9	0	0	0	0	0	3.8	0	0	0	1.3	0	0
1,048.0	1,060.0	17.3	0	156.9	352.1	0	0	0	0	0	14.4	3.3	0	0	3.6	0	6.6
1,060.0	1,065.0	27.5	2.3	21.0	57.2	0	0	0	1.5	1.0	9.2	2.1	0	0	0	0	9.1
1,065.0	1,071.0	71.1	5.3	8.5	11.4	0	1.4	0	2.2	1.9	13.7	3.6	0	0	0	0	18.8
1,071.0	1,190.0	504.6	1,763.0	1,023.0	1,539.0	1,178.0	1,661.0	926.1	1,393.0	1,106.0	1,118.0	890.3	916.8	96.3	1,026.0	0	371.3
1,190.0	1,225.0	0	0	329.0	551.5	202.5	11.3	514.0	791.5	1,653.0	533.9	6.2	636.0	473.9	6.6	0	0
1,225.0	1,250.0	0	0	13.3	13.5	1,702.0	0	101.4	257.0	370.2	30.0	0	128.6	1,317.0	5.7	0	0
1,250.0	1,301.0	0	0	0	172.4	204.6	45.9	619.8	423.6	236.3	693.8	0	0	551.6	12.3	0	0
1,301.0	1,307.0	0	0	0	0	5.6	32.8	9.7	126.8	155.8	170.9	0	0	14.9	0	0	0
1,307.0	1,397.0	0	0	19.1	18.7	251.0	236.0	0	283.2	0	194.7	60.2	94.1	68.6	148.6	0	32.0
1,397.0	1,420.0	0	0	0	0	0	0	0	24.6	0	32.4	12.9	0	154.9	128.9	0	0
1,420.0	1,535.0	0	0	0	0	0	0	0	0	0	0	0	0	169.9	0	0	0

Units for absorbance bands are atm⁻¹ cm⁻² STP.

CFC-113 and CFC-114) show significant absorption in the region 934 to 1,070 cm⁻¹, which overlaps with the infrared absorption bands of ozone.

Tables 3 and 4 detail intermediate results for calculation of HGWP. Table 3 shows the change in net infrared radiative flux at the tropopause (at 12 km) for tropospheric concentrations of 1 p.p.b.v. (part per 10⁹ by volume) as calculated by each model. Note that each of the model's calculations was based on different cloud assumptions (Du Pont used fixed 50% grey (albedo = 0.5) cloud cover, whereas AER used non-grey cloud with 48.5% cover). Table 4 shows the resulting values for specific surface temperature increases for each compound. Model results correlate with each chemical's total band strength, with compensation made for overlapping absorption by other active gases in the infrared and for differences in the chemical profile. The inter-model differences are consistent with previously reported patterns³, arising mainly from different treatments of tropospheric feedbacks.

TABLE 3 Net infrared radiative flux change at 12 km due to 1-p.p.b.v. tropospheric mixing ratio

Species	AER	Du Pont
CFC-11	0.175	0.133
CFC-12	0.248	0.158
CFC-113	0.223	0.163
CFC-114	0.258	0.181
CFC-115	0.204	0.164
HCFC-22	0.151	0.107
HCFC-123	0.140	0.092
HCFC-124	0.153	0.108
HFC-125	0.189	0.119
HFC-134a	0.135	0.095
HCFC-141b	0.109	0.076
HCFC-142b	0.144	0.101
HFC-143a	0.111	0.087
HFC-152a	0.092	0.059
CCl ₄	0.080	0.063
CH ₃ CCl ₃	0.038	0.033
[2 × CO ₂]	4.41	3.87

Units are W m⁻².

The effects of the various feedback assumptions can be accounted for by normalizing the surface warming to the calculated surface warming from a doubling of carbon dioxide in the manner shown in footnote of Table 4. The normalized results, shown in the final two columns of Table 4, show good agreement between the two model results. All results are credible to two significant figures at best.

Also included in Table 4 are tabulations of the climate feedback factor, λ , which is the ratio of the model-calculated surface-temperature change to the perturbation in the net radiative forcing. As can be seen, these values are consistent for each of the models, thereby validating the assumptions made for the equivalent forms of the HGWP definition.

Whereas the total infrared band strengths are, on average, comparable among species, both the net infrared flux and the specific surface-warming values for HCFCs and HFCs are, on average, lower than the average values for CFCs by ~40%. This lower value results from the fact that most hydrogenated halocarbons have bands that overlap the bands of both CH₄ and N₂O (wavenumber > 1,190 cm⁻¹) as well as water vapour, unlike CFC-11 and CFC-12.

Because the total infrared band strengths for halocarbons are of the same order of magnitude (within a factor of three), the dT_s values have a similar range. However, once atmospheric lifetime factors are used to calculate HGWP values, the spread among chemicals is more pronounced.

As HGWP values vary linearly with lifetimes, it is critical that the HGWP values are based on an appropriate common reference set of lifetimes. Reference lifetimes for CFCs are based on the estimates used in model calculations done for the World Meteorological Organization⁸ report. Reference lifetimes for HCFCs and HFCs are from the analysis of M. J. Prather in ref. 9. He calculated OH concentration fields using a three-dimensional model for tropospheric processes and then calculated lifetimes of HCFCs and HFCs. The values reported were obtained by scaling the results by the ratio of the empirically determined lifetime of 6.3 years for methylchloroform to the model-calculated lifetime. These predicted lifetimes agree well with lifetime predictions using radiocarbon analyses¹⁰. As seen in Table 5, HGWP results from the two models agree well.

Furthermore, the HGWP values for fully halogenated CFCs range from 1.0 to 7.5, whereas the HCFC and HFC values range

TABLE 4 Specific surface-temperature increases (warming resulting from 1 p.p.b.v. of each gas)

Species	Modelled warming (K per p.p.b.v.)		λ (K per p.p.b.v. per W m^{-2})		Normalized warming* (K per p.p.b.v.)	
	AER	Du Pont	AER	Du Pont	AER	Du Pont
CFC-11	0.135	0.084	0.771	0.632	0.088	0.102
CFC-12	0.202	0.102	0.815	0.647	0.131	0.124
CFC-113	0.174	0.103	0.780	0.632	0.113	0.125
CFC-114	0.208	0.115	0.806	0.635	0.135	0.139
CFC-115	0.170	0.107	0.833	0.652	0.110	0.130
HCFC-22	0.124	0.070	0.821	0.650	0.081	0.084
HCFC-123	0.111	0.059	0.793	0.644	0.071	0.072
HCFC-124	0.126	0.070	0.824	0.645	0.082	0.084
HFC-125	0.160	0.078	0.847	0.654	0.104	0.094
HFC-134a	0.114	0.061	0.844	0.647	0.074	0.074
HCFC-141b	0.086	0.048	0.789	0.637	0.056	0.059
HCFC-142b	0.120	0.066	0.833	0.651	0.078	0.080
HFC-143a	0.092	0.054	0.829	0.625	0.060	0.066
HFC-152a	0.076	0.038	0.826	0.649	0.049	0.046
CCl_4	0.062	0.040	0.775	0.628	0.040	0.048
CH_3CCl_3	0.027	0.020	0.710	0.618	0.018	0.025

* Normalized by: $dT_s \times 2 \text{ K} / (dT_s \text{ for } 2 \times \text{CO}_2)$ where dT_s for $2 \times \text{CO}_2$ is 3.08 K using the AER model and 1.651 K using the Du Pont model.

TABLE 5 HGWPs based on reference lifetimes

Species	Reference lifetime	AER	Du Pont
CFC-11	60.0	1.0	1.0
CFC-12	120.0	3.4	2.8
CFC-113	90.0	1.4	1.4
CFC-114	200.0	4.1	3.7
CFC-115	400.0	7.5	7.6
HCFC-22	15.3	0.37	0.34
HCFC-123	1.6	0.020	0.017
HCFC-124	6.6	0.10	0.092
HFC-125	28.1	0.65	0.51
HFC-134a	15.5	0.29	0.25
HCFC-141b	7.8	0.097	0.087
HCFC-142b	19.1	0.39	0.34
HFC-143a	41.0	0.76	0.72
HFC-152a	1.7	0.033	0.026
CCl_4	50.0	0.34	0.35
CH_3CCl_3	6.3	0.022	0.026

Lifetimes for CFCs are based on estimated lifetimes used in ref. 8. Lifetimes for HCFCs and HFCs are based on Prather⁹.

from 0.02 to 0.7. Of the hydrofluorocarbons, HCFC-22 and HFC-125 and HFC-143a have the largest HGWP values. It is important to recognize that these compounds may be replacing compounds with HGWP values of ~ 3 , so that improvements must be judged relative to the compound that is being replaced.

Uncertainties

Uncertainties in the calculations of the effect of these gases on global warming fall into two general categories: those generalized to the total greenhouse effect and those specific to the individual species considered.

There are a number of problems that need to be resolved in the modelling of greenhouse warming. The radiative properties of the Earth's surface, such as changes in the surface and

ice-cover albedos, and changes in cloud cover and composition need to be quantified as a function of surface warming. Changes in the temperature structure of the atmosphere will affect the convective patterns and chemistry of the stratosphere. The coupling of oceans (as heat reservoirs) and ocean currents to surface-temperature changes will also affect the timing and location of the warming. Research is being carried out worldwide to understand these questions, which apply to all trace gases that affect the future radiative balance of the Earth.

Uncertainties also exist concerning the individual radiative effects of each species (CFCs, HCFCs and HFCs). The temperature dependence of the absorbances of individual species needs to be resolved and parametrized for use in climate models. The chemical processes affecting both lifetimes and atmospheric profiles are also the subject of continuing research.

Resolution of questions related to the general greenhouse warming will directly affect the modelled timing and magnitude of global warming, but will not affect the relative contributions by individual gases. On the other hand, resolution of the radiative and chemical parameterizations for individual halocarbons will directly affect the HGWP values for each species.

Applications

The reduction in HGWP that might be expected from the replacement of a CFC by a hydrohalocarbon can be estimated by taking the ratio of the HGWP of the hydrohalocarbon to the HGWP of the CFC it would replace. For example, the HGWP of HCFC-134a is only 8.7% as large as that of CFC-12 ($0.27/3.1 = 0.087$, based on averaged HGWP values). Of course, the relative quantities of the compound required in the particular application must also be taken into account.

For new or extended uses of these replacement compounds, it must be recognized that the contribution to warming by all of these substances is proportional to their atmospheric concentrations. Significant radiative perturbations may therefore result from unbounded use. Contributions, along with transient responses, can be easily estimated from HGWP parameters and corresponding chemical lifetimes. $\square$

Received 30 August 1989; accepted 29 January 1990.

1. Fisher, D. A. et al. *Nature* **344**, 508–512 (1990).
2. Wang, W.-C. & Molnar, G. *J. geophys. Res.* **90**, 12971–12980 (1985).
3. Owens, A. J. et al. *J. geophys. Res.* **90**, 2283–2311 (1985).
4. Wang, W.-C., Molnar, G., Ko, M. K. W., Goldenberg, S. & Sze, N. D. *Tellus* **42b**, 149–161 (1990).
5. Kagann, R. H., Elkins, J. W. & Sams, R. L. *J. geophys. Res.* **88**, 1427–1432 (1983).
6. Varanasi, P. & Chudamani, S. *J. geophys. Res.* **93**, 1666–1668 (1988).
7. Rogers, J. D. & Stephens, R. D. *J. geophys. Res.* **93**, 2423–2428 (1988).
8. World Meteorological Organization, *Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20* (Geneva, 1989).

9. Prather, M. J. in *World Meteorological Organization, Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20* Vol. II, 149, Appendix: AFEAS Report (Geneva, 1989).

10. Derwent, R. G. & Volz-Thomas, A. in *World Meteorological Organization, Scientific Assessment of Stratospheric Ozone: 1989, Global Ozone Research and Monitoring Project—Rep. No. 20* Vol. II, 127, Appendix: AFEAS Report (Geneva, 1989).

ACKNOWLEDGEMENTS. The authors thank the following companies: Allied-Signal Corporation, Atochem, E. I. Du Pont de Nemours and Co., Hoechst Aktiengesellschaft, ICI Chemicals and Polymers Limited, and Montefluos SPA.

Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation

Bernhard Lüscher*, Erik Christenson*, David W. Litchfield†, Edwin G. Krebs† & Robert N. Eisenman*

* Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104, USA

† Howard Hughes Medical Institute, University of Washington, School of Medicine, Seattle, Washington 98195, USA

The c-Myb nuclear oncoprotein is phosphorylated *in vitro* and *in vivo* at an N-terminal site near its DNA-binding domain by casein kinase II (CK-II) or a CK-II-like activity. This *in vitro* phosphorylation reversibly inhibits the sequence-specific binding of c-Myb to DNA. The site of this phosphorylation is deleted in nearly all oncogenically activated Myb proteins, resulting in DNA-binding that is independent of CK-II. Because CK-II activity is modulated by growth factors, loss of the site could uncouple c-Myb from its normal physiological regulator.

THE c-*myb* proto-oncogene encodes a highly conserved nuclear phosphoprotein (c-Myb) of relative molecular mass 75,000–89,000 (M_r 75–89K) that has a short half-life and is normally expressed in immature haematopoietic cell lineages^{1–5}. The expression of c-Myb is linked to the differentiation state of the cell, because expression is downregulated during terminal differentiation of haematopoietic cells, and constitutive expression of introduced c-*myb* blocks the induced differentiation of erythroleukaemia cells^{6–9}. The v-*myb*-containing avian acute leukaemia viruses AMV (avian myeloblastosis virus) and E26 also seem to interfere with monocyte-macrophage differentiation^{10–12}. These viruses encode a v-Myb protein that is an N- and C-terminally truncated version of c-Myb¹³. Oncogenically activated c-*myb* genes in murine myelogenous leukaemias and avian bursal lymphomas have similar deletions, particularly in the 5' region^{14–17}. All the oncogenically activated Myb proteins retain a well-defined DNA-binding domain consisting of a 52-amino-acid imperfect repeat^{2,18–20} that mediates the specific interaction of Myb with a hexameric nucleotide sequence²¹. In addition, both v-Myb and c-Myb seem to function as transcriptional activators when tested on promoters containing the specific binding sequence^{22–25}. In addition to the DNA-binding domain, other Myb domains involved in transcriptional activation and nuclear localization have been defined. These regions are not deleted during the oncogenic conversion of c-*myb*^{18,19,22,24}. We were interested in the possibility that the protein segments consistently deleted during oncogenic activation represent regions through which cellular signals regulating the function of Myb operate.

There is much evidence that protein phosphorylation has key roles in regulating the cell cycle, transcription^{26,27}, and signal transduction (for reviews, see refs 28–30). A protein kinase thought to be involved in mitogenesis is CK-II, a ubiquitous highly conserved kinase, located in both nucleus and cytoplasm, with a strong specificity for Ser and Thr residues lying in clusters of acidic amino acids (ref. 31; for reviews, see refs 29, 32). CK-II activity is modulated after treating different cell types with growth stimulatory factors^{33–37}. Two proteins implicated in neoplastic transformation are phosphorylated by CK-II: the Myc oncoprotein and the E7 protein encoded by human papillomavirus type 16^{38,39}. Also, Myb contains several conserved

potential consensus sites for CK-II phosphorylation. Here we show that Myb is phosphorylated *in vivo* and *in vitro* at a site near to its DNA-binding domain by CK-II or a CK-II-like activity. Phosphorylation at this site negatively regulates specific binding of Myb to DNA. The N-terminal truncations of oncogenically activated Myb proteins involve deletion of the phosphorylation site. We find that such deletions result in DNA-binding that is independent of CK-II phosphorylation.

Site-specific phosphorylation of Myb by CK-II

To determine whether Myb is a substrate for CK-II, we prepared Myb-containing immunocomplexes from the chicken B cell line BK3A using a well-characterized antiserum (anti-Bp52) raised against the DNA-binding domain of v-Myb^{2,40}. The immunoprecipitates were incubated with purified CK-II and [γ -³²P]ATP, and fractionated by SDS-PAGE. Labelled phosphate was incorporated into the β -subunit of CK-II and into a 75K protein which co-migrated with [³⁵S]methionine-labelled c-Myb (Fig. 1a, lanes 1 and 4). No labelled phosphate was transferred to the 75K Myb protein when CK-II and [γ -³²P]ATP were incubated without the Myb immunocomplex, when the Myb immunocomplex was incubated without CK-II (Fig. 1a, lanes 2 and 3), or when precipitation was blocked by an excess of the Bp52 Myb protein fragment originally used as immunogen (data not shown). That this *in vitro* reaction of CK-II with p75^{c-myb} is specific is indicated by the findings that phosphorylation of Myb was blocked by a competitive inhibitor of CK-II (Fig. 1a, lane 5), and that the immunoglobulin heavy chain which is present in relatively large amounts in the immunocomplex was not phosphorylated by CK-II (Fig. 1a), confirming that CK-II does not act profligately^{31,38}.

We then investigated whether the region(s) in Myb that is phosphorylated *in vivo* is the same as that phosphorylated *in vitro* by CK-II. Figure 1b (lane 1) shows anti-Bp52 immunoprecipitation of radioactive p75^{c-myb} from a lysate of ³²P-labelled BK3A cells. Phospho-amino acid analysis indicated that only Ser residues were phosphorylated *in vivo* or by CK-II *in vitro* (data not shown). When the *in vivo* ³²P-labelled p75^{c-myb} was eluted from the gel, digested with trypsin and subjected to thin layer electrophoresis and chromatography, we detected five phosphopeptides (Fig. 2a, left). Two of those labelled *in vivo* (Fig. 2a; left, arrows) seem to behave identically to the only two tryptic phosphopeptides detected after CK-II phosphorylation of c-Myb *in vitro* (Fig. 2a; middle, arrows) because they co-migrated after mixing of the two tryptic digests (Fig. 2a; right, arrows). Similar results were obtained with c-Myb from 70Z murine pre-B cells (data not shown). Phosphorylation *in vitro* by CK-II therefore seems to occur in the same regions as do a subset of the *in vivo* phosphorylations for avian and murine c-Myb.

To establish the site(s) of CK-II phosphorylation in c-Myb we used synthetic peptides comprising candidate c-Myb CK-II consensus regions: one in the DNA-binding domain (peptide Myb^{143–152}, comprising Myb residues 143–152) and the other proximal to the N terminus (peptide Myb^{2–20}; see legend to Fig. 2 for sequence, and Fig. 6 for location of potential CK-II

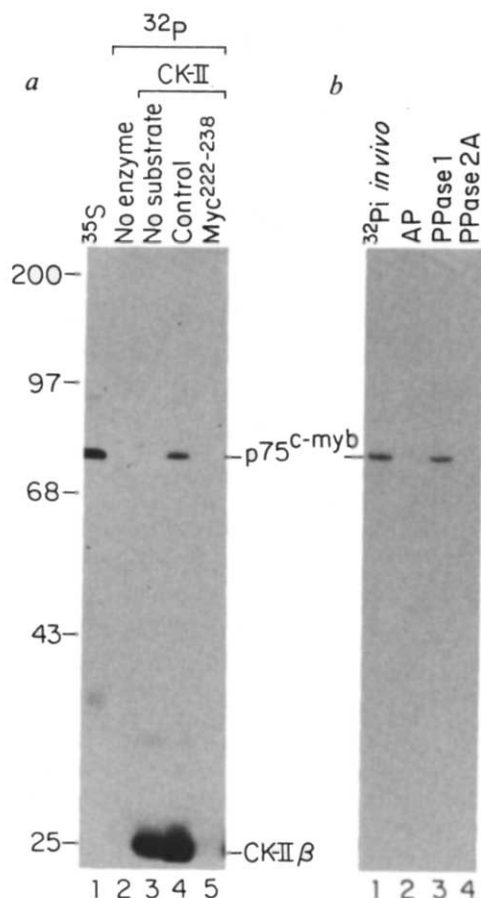


FIG. 1 *a*, Phosphorylation of Myb by CK-II. Myb was immunoprecipitated from [^{35}S]methionine-labelled BK3A cells (lane 1) or unlabelled cells (lanes 2, 4, 5). Immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of [γ - ^{32}P]ATP without enzyme (lane 2), with CK-II (lane 4) or with CK-II in the presence of 1 mM Myc $^{222-238}$ peptide (lane 5, comprises Myc residues 222–238). Autophosphorylation of CK-II without exogenous substrate is shown in lane 3. Samples were analysed by electrophoresis on a 10% SDS-polyacrylamide gel. *b*, Sensitivity of *in vivo* phosphorylated Myb to protein phosphatases. BK3A cells were labelled with [^{32}P]orthophosphate (^{32}Pi), and Myb immunoprecipitated. The immunoprecipitates were mock-treated (lane 1) or treated with alkaline phosphatase (AP, lane 2), protein phosphatase type 1 (PPase 1, lane 3), or protein phosphatase type 2A (PPase 2A, lane 4). Samples were analysed by electrophoresis on a 10% SDS-polyacrylamide gel.

METHODS. BK3A cells (a chicken bursal lymphoma), the anti-Myb antiserum (anti-Bp52), [^{35}S]methionine labelling and immunoprecipitation were as described in ref. 40. The peptide Myc $^{222-238}$ is a low- K_m substrate for CK-II 38 . For kinase assays, immunoprecipitates from unlabelled cells were washed twice in PBS after the final RIPA buffer 40 wash. The CK-II kinase reactions 38 were performed in 15 μl kinase buffer (30 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl_2 , 4.8 μM [γ - ^{32}P]ATP at 300 Ci mmol^{-1}) with 1 μl CK-II purified from bovine testes (0.36 mg ml^{-1} ; specific activity, 2,800 nmol min^{-1} mg^{-1} with RRRDDSDDD (single-letter amino-acid code) as a substrate; D.W.L., manuscript in preparation) for 30 min at 30 °C. The reactions were stopped by boiling the samples in SDS-PAGE sample buffer. Labelling with ^{32}P was performed as described 38 . For alkaline phosphatase treatment, anti-Bp52 immunoprecipitates of ^{32}P -labelled Myb were washed first with RIPA buffer then twice in 100 mM Tris-HCl, pH 8.0, 50 mM MgCl_2 , 1% aprotinin, then resuspended in 45 μl same buffer, and 5 μl alkaline phosphatase (Sigma, 0.14 U μl^{-1}) added. For PPase-1 and -2A treatment, immunoprecipitates were washed twice in 20 mM imidazole, pH 7.2, 0.1% β -mercaptoethanol, 1 mg ml^{-1} BSA, 1 mM EDTA after the final RIPA buffer wash. Immunoprecipitates were then resuspended in 20 μl of the same buffer, and either 2 μl PPase 1 (7,500 nmol min^{-1} ml^{-1} versus phosphorylase *a*) or 2 μl PPase 2A (1,400 nmol min^{-1} ml^{-1} versus phosphorylase *a*) added. Phosphatase treatments were performed at 30 °C for 30 min. The following prestained M_r size markers (left, $M_r \times 10^{-3}$; BRL) were used: myosin (200K), phosphorylase b (97.4K), BSA (68K), ovalbumin (46K) and α -chymotrypsinogen (25.7K). CK-II- β : β -subunit of CK-II.

phosphorylation sites in c-Myb). The Myb $^{2-20}$ peptide was an excellent substrate for CK-II phosphorylation, which was on serine, and had a Michaelis constant (K_m) of 13 μM and involved the incorporation of 1.5 mol phosphate per mol peptide; phosphorylation of the Myb $^{143-152}$ peptide, however, had a poor K_m and was on threonine. To determine which serines were phosphorylated, the Myb $^{2-20}$ peptide was phosphorylated with CK-II, derivatized and sequenced 41,42 . The results demonstrated

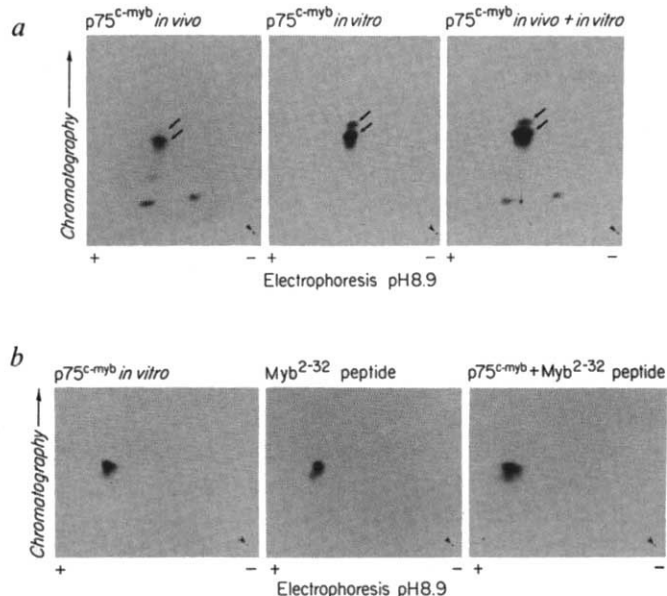


FIG. 2 *a*, Comparison of *in vivo* and *in vitro* CK-II-phosphorylated Myb by two-dimensional phosphopeptide mapping. Left panel, tryptic phosphopeptide pattern of *in vivo* labelled Myb; middle panel, *in vitro* CK-II-labelled Myb; right panel, mixture of the two samples. Arrows, two CK-II-specific peptides. *b*, Comparison of Myb and a peptide encompassing amino acids 2–32 of chicken Myb (Myb $^{2-32}$) after phosphorylation by CK-II *in vitro*. Protein and peptide were first digested with trypsin and then with thermolysin before analysis in two dimensions. Left and middle panels, peptides resulting from the double digest of Myb or Myb $^{2-32}$ peptide phosphorylated by CK-II, respectively. Right panel, two samples mixed. Note that if c-Myb was phosphorylated at the only other Ser (residue 8) in the region corresponding to Myb $^{2-32}$, the ^{32}P label would not have co-migrated with the peptide, because thermolysin cleaves the peptide between Ser 8 and the CK-II phosphorylation site. The Myb $^{143-152}$ peptide was a relatively poor substrate for CK-II phosphorylation ($K_m = 2$ mM). Also, this peptide was phosphorylated only on Thr, whereas c-Myb was phosphorylated exclusively on Ser. Therefore it seems unlikely that the candidate site in the DNA-binding domain is a target for CK-II phosphorylation. The mapping experiments with the Myb $^{2-32}$ peptide also argue against the possibility that a third potential CK-II phosphorylation site, located at residues 580–583, is phosphorylated by CK-II *in vitro*.

METHODS. Myb was phosphorylated as described in the legend to Fig. 1. The protein was extracted from the gel in 50 mM NH_4HCO_3 , pH 7.4, 0.1% SDS, 1% β -ME, and precipitated in 15% trichloroacetic acid using 10 μl ml^{-1} RNase A as carrier 38 . For peptide phosphorylation, 3.6 ng CK-II was added to 10 μl of a 50 μM solution of Myb $^{2-32}$, (ARRPRHSIYSSDDDEEDVEMYDHDYDGLLPK; single-letter amino-acid code) in CK-II assay buffer. The reaction was performed at 30 °C for 60 min. The reaction mix was spotted onto a cellulose thin-layer plate (Merck), and the peptide was separated from other labelled components of the reaction by electrophoresis at pH 3.5, 1.6 kV for 20 min. The peptide was extracted from the cellulose with 50 mM NH_4HCO_3 and lyophilized. Precipitated protein, as well as lyophilized peptide, were oxidized in performic acid (formic acid/hydrogen peroxide, 10:1) for 90 min, lyophilized and digested twice with 10 μg TPCK (tosyl-phenyl-chloromethyl-ketone)-treated trypsin (Cooper Biomedicals) in 50 mM NH_4HCO_3 , pH 7.4, at 37 °C for 24 h. For double digests, trypsinized samples were digested twice with 10 μg thermolysin (Boehringer) in 50 mM NH_4HCO_3 , pH 7.4, at 55 °C for 24 h. Peptides were then lyophilized twice in H_2O and separated on cellulose thin-layer plates by electrophoresis at pH 8.9, 1.2 kV for 20 min in the first dimension, and by ascending chromatography (*n*-butanol/pyridine/acetic acid/water, 15:10:3:12) in the second dimension 38,50 .

that the two serine residues at positions 11 and 12 were phosphorylated to equal molarity (data not shown, see Fig. 6).

For mapping studies, a larger N-terminal peptide, Myb²⁻³², was radioactively phosphorylated with CK-II, digested with trypsin and thermolysin, and its two-dimensional map compared with that of *in vitro* phosphorylated p75^{c-myb}. Double digestion of either the Myb²⁻³² peptide or p75^{c-myb} (Fig. 2b, left and middle) generated a single phosphopeptide from each that co-migrated with the other when the two digests were mixed (Fig. 2b, right). Therefore our mapping experiments indicate that Ser 11 and Ser 12, lying in a functional CK-II recognition sequence located at residues 11–18 of p75^{c-myb}, were phosphorylated *in vivo* and by CK-II *in vitro*.

Phosphorylation regulates specific DNA-binding

We examined the effects of CK-II phosphorylation on the ability of Myb to specifically bind to DNA. To directly assay DNA-binding activity of Myb proteins from different sources we developed a method using Myb derived from immunoprecipitates. Unlabelled cell lysates were immunoprecipitated with anti-Myb antiserum and the resulting immunocomplexes disrupted by incubation with a chaotropic agent. After dialysis, the proteins were treated with different agents, mixed with ³²P-labelled oligonucleotide probes, and the extent of binding determined by retardation of the probe on nondenaturing polyacrylamide gels^{43,44}. Among the oligonucleotides used (see Fig. 3a for sequences) were those containing one or two copies of the previously defined Myb response element (1× or 2× MRE).

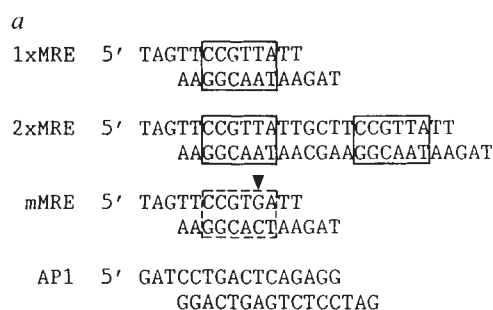
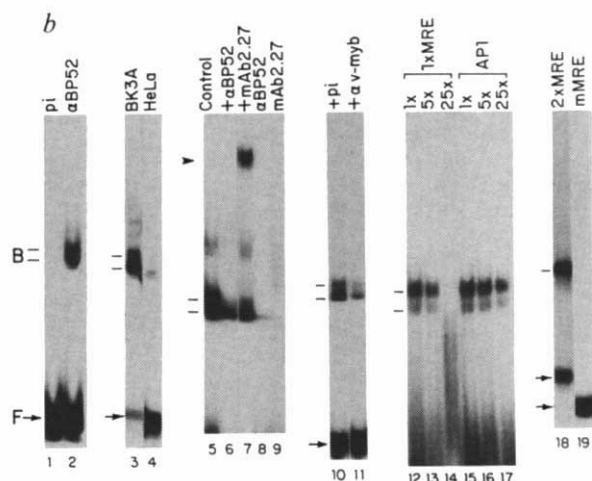


FIG. 3 a, Double-stranded oligonucleotides used for gel mobility shift assay containing one or two MREs and a mutant MRE (1×MRE, 2×MRE and mMRE, respectively). The mMRE has a single base-pair change compared with 1×MRE (arrowhead). A control oligonucleotide containing an AP-1-binding site is also shown. The Myb-binding sequences are boxed; the mutant site is boxed with a broken line. **b**, Myb protein binds specifically to double-stranded oligonucleotides containing an MRE. Myb was immunoprecipitated from BK3A cells with anti-Myb antiserum anti-Bp52, denatured, and renatured, as described below. This protein was then used for gel mobility shift assays (lanes 2, 3, 5–7, 10–19). In addition, Myb prepared using monoclonal antibody mAb2.27 (prepared against a bacterial fusion protein; mAb2.27 recognizes an epitope in v-Myb outside the DNA-binding domain⁵¹) for the immunoprecipitation also retarded MRE oligonucleotides (data not shown). Immunoprecipitations from BK3A cells using preimmune serum (PI, obtained from rabbit immunized with the Bp52 fusion protein), or from HeLa cells using anti-Bp52, were used for lanes 1 and 4, respectively. Anti-Bp52 (1 µl), 1 µl mAb2.27 culture supernatant (specific for chicken Myb⁵¹), 1 µl preimmune serum or 1 µl anti-v-Myb serum (rabbit polyclonal sera raised against a bacterial fusion protein⁵²) were added to renatured Myb immunoprecipitates in lanes 6, 7, 10 and 11, respectively. Anti-Bp52 (1 µl) and 1 µl mAb2.27 alone were analysed for binding to 1×MRE in lanes 8 and 9, respectively. As competitors, unlabelled 1×MRE (lanes 12–14) or AP-1 (lanes 15–17) were used at a onefold, fivefold or 25-fold molar excess over the labelled probe. The ³²P-labelled probes were as follows: 1×MRE (lanes 1, 2, 5–17), 2×MRE (lanes 3, 4, 18) or mMRE (lane 19). Arrowhead, position of the probe after mAb2.27 addition; F, free probe; B, bound probe. **METHODS**. Oligonucleotides were purified over a NAP-5 column (Pharmacia). Corresponding strands were hybridized and labelled with [³²P]dCTP, dATP, dTTP using the Klenow fragment of DNA polymerase (specific activities, 5–20 × 10⁴ c.p.m. ng⁻¹). Between 0.1 and 1 ng gel-purified labelled oligonucleotides were added per sample. The proteins used for the gel mobility

We established that the binding reaction required Myb by performing a series of control experiments. Proteins derived from immunocomplexes formed with anti-Myb, in the absence of antigen, failed to bind the ³²P-labelled MRE probe (Fig. 3b, lanes 8 and 9). But when the MRE was incubated with proteins from an anti-Myb (anti-Bp52) immunoprecipitate from a BK3A cell lysate, significant binding of the probe was detected (Fig. 3b, lanes 2, 3, 5). By contrast, incubation with (1) an anti-Bp52 immunoprecipitate from cells with no detectable c-Myb (HeLa), (2) an immunoprecipitate from BK3A cells using preimmune serum, or (3) an immunocomplex containing c-Myc protein, resulted in little or no binding to the MRE (Fig. 3b, lanes 1–4; and data not shown). Also, when the renatured proteins from an anti-Bp52 immunocomplex were treated with fresh anti-Bp52 (which reacts with the Myb DNA-binding domain), or with anti-v-Myb, binding of the probe was greatly diminished. Treatment with an anti-Myb monoclonal antibody (mAb2.27) resulted in a large reduction in mobility of most of the bound MRE probe (Fig. 3b, lanes 6, 7 and 11). Treatment with preimmune serum had no effect on Myb DNA-binding (Fig. 3b, lane 10). These experiments indicate that Myb protein in the renatured immunocomplex is involved in the specific retardation of the MRE.

The specificity of the binding of Myb to the MRE was confirmed by probe competition experiments. When increasing amounts of unlabelled 1×MRE or an oligonucleotide containing an AP-1-binding site (Fig. 3a) were added to the Myb binding reaction in the presence of the ³²P-labelled MRE, only the unlabelled MRE was able to abolish binding at a 25-fold



shift assays were prepared as follows: Cells were grown in spinner-flasks to a density of 1–2 × 10⁶ ml⁻¹. The cells from 1 litre of culture were collected, washed in PBS and then lysed in 8 ml PBS containing 0.5% Nonidet P-40, 0.5% deoxycholate and 1% aprotinin for 5 min on ice. The lysates were cleared by centrifugation for 10 min at 12,000 r.p.m. in a Sorval SS34 rotor and the supernatants incubated with the indicated antiserum. The immunocomplexes were collected using protein A-Sepharose 4B beads (Sigma), washed four times in lysis buffer and twice in PBS. The proteins in the immunocomplex were then denatured and extracted from the sepharose beads with 2 × 200 µl 6 M guanidine-HCl, 0.1% β-mercaptoethanol (20 min each) at room temperature. The proteins were renatured by dialysing the samples against gel mobility shift buffer (20 mM HEPES buffer, pH 7.5, 50 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM β-mercaptoethanol, 8% (v/v) glycerol, 0.1% aprotinin, 0.1% Na₃S₂O₄). The dialysed samples were stored for up to 2 days at 4 °C, or for longer in liquid nitrogen. Gel mobility shift reactions^{43,44} contained 5–9 µl protein sample (10 µl total volume). Each sample was adjusted to 1 × gel mobility shift buffer conditions. In experiments in which additional components were added to the reactions, control samples were also adjusted to the altered buffer conditions. The gel mobility shift reactions were performed at 25 °C for 20 min, and the samples were analysed on nondenaturing 5% polyacrylamide gels in TBE (50 mM Tris-buffer, 50 mM boric acid, 1 mM EDTA). Each group of lanes were from the same experiment and the same gel.

excess over the labelled probe, whereas the AP-1 competitor had little effect (Fig. 3b, lanes 12–17). Similarly, a 1,000-fold excess of poly(dI). poly(dC) had no effect on the binding of Myb to the MRE (data not shown). In addition, we tested a mutant MRE (mMRE, Fig. 3a) containing a nucleotide change that abolishes Myb-binding in a DNase protection assay²¹. The labelled mMRE did not bind Myb in our assay, and a 40-fold excess of the mMRE failed to compete with the 1XMRE probe for the binding of Myb (Fig. 3b, lanes 18 and 19; and data not shown).

These data demonstrate that the immunocomplex binding assay can be used to monitor the specific interaction of Myb with the MRE. In addition, purified CK-II can be used to reversibly phosphorylate Myb in an immunocomplex (Fig. 1). We therefore used the assay to determine the effect of CK-II phosphorylation on the specific interaction of Myb with the MRE. When an anti-Bp52 immunoprecipitate from BK3A cells was denatured, renatured, and incubated with ATP, or with heat-inactivated CK-II and ATP, we detected binding to the labelled MRE probe (Fig. 4, lanes 1 and 2). But when active CK-II with ATP was used in the reaction, Myb binding to the MRE was almost completely abolished (Fig. 4, lane 3). The inhibition of binding was dependent on the concentration of CK-II and the presence of ATP (Fig. 4, lanes 4–9). Also, pretreatment of the immunocomplex with alkaline phosphatase or phosphatase 2A induced a small increase in the DNA-binding, indicating that removal of phosphate from endogenously occupied sites can positively affect specific binding (Fig. 1b, lanes 2–4; Fig. 4, lanes 10 and 12, and 14 and 16). Nonetheless, subsequent treatment with CK-II abolished the binding (Fig. 4, lanes 10–17). To determine whether CK-II treatment leads to loss of DNA-binding activity by irreversibly denaturing Myb, we treated the CK-II-phosphorylated Myb with protein phosphatase 2A, an enzyme that reverses CK-II-mediated phosphorylation and removes *in vivo* phosphate from Myb (Fig. 1b, lanes 2–4, and data not shown). About 80% restoration of DNA-binding activity can be obtained with CK-II-phosphorylated Myb after phosphatase treatment (Fig. 4, lanes 14–18).

These data indicate that phosphorylation by CK-II inhibits specific DNA-binding by Myb in a reversible manner.

Oncogenic activation of Myb

With only two exceptions, every known oncogenically activated *c-myb* gene encodes truncated proteins which, although possessing N-, and occasionally, C-terminal deletions to varying extents, lack the CK-II phosphorylated region but retain the DNA-binding domain (refs 13–17, 45; W. Hayward and E. Humphries, personal communication). To test the DNA-binding activity of such proteins we prepared immunocomplexes from an AMV-transformed chicken myeloblastic cell line, which produces high levels of N- and C-terminally truncated v-Myb. Both c-Myb and v-Myb proteins bind to DNA in the absence of CK-II or in the presence of CK-II without ATP (Fig. 5, lanes 1, 2, 4 and 5). On incubation with CK-II and ATP, binding of c-Myb to the MRE is inhibited, as expected, but the binding activity of the AMV v-Myb protein is unaffected relative to control (Fig. 5, lanes 1–6). In accordance with AMV v-Myb containing a deletion in the region containing the CK-II phosphorylation site, we could not detect incorporation of phosphate into this protein after CK-II treatment *in vitro* (data not shown).

To extend our observations on the effects of CK-II phosphorylation to more subtly rearranged c-Myb proteins, we examined the DNA-binding activity of normal murine c-Myb, and *c-myb*-encoded proteins of two murine leukaemia virus (MLV)-induced myelogenous leukaemias, MML7 and MML9. Both MML7 and MML9 synthesize smaller *c-myb*-encoded proteins owing to MLV insertions between *c-myb* exons UE1-VE1 and UE2-UE1 respectively (refs 45, 46; see Fig. 6). Both of these insertions result in loss of the CK-II phosphorylation region. Normal c-Myb protein from the 70Z pre-B cell line specifically bound to the labelled MRE probe. As expected, MRE-binding was blocked after treatment with CK-II with ATP (Fig. 5, lanes 7–9). The MML7 and MML9 Myb proteins also bound to the MRE probe, but to a lesser extent, because the levels of Myb were lower in MML7 and MML9 cells than in 70Z or avian cells (data not shown). Importantly, CK-II treatment failed to inhibit

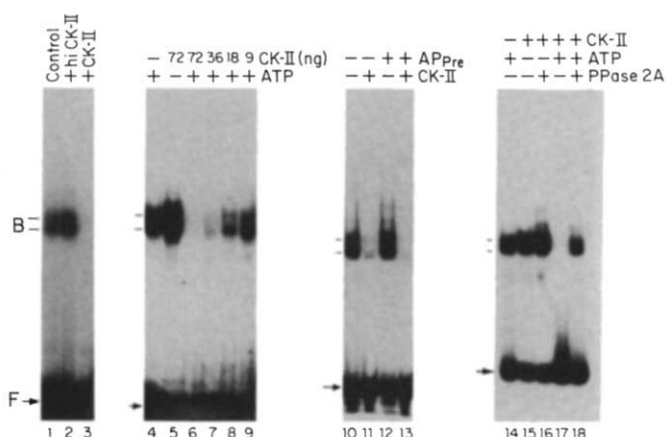


FIG. 4 Phosphorylation of Myb by CK-II inhibits DNA-binding. Myb was partially purified from BK3A cells as described in the legend to Fig. 3 and used without further treatment in gel mobility shift assays (lanes 1, 4, 14). Immunoprecipitated Myb was either mock-treated (lanes 10, 11) or treated with alkaline phosphatase before the denaturing-renaturing step (AP_{pre}, lanes 12, 13). Partially purified Myb was preincubated with 72 ng heat-inactivated CK-II (hiCKII, lane 2), 72 ng active CK-II (lanes 3, 5, 6, 11, 13) or with 36 ng (lane 7), 18 ng (lanes 8, 15–18), 9 ng (lane 9) active CK-II. ATP was added to 0.5 mM (lanes 1–4, 6–13) or to 0.01 mM (lanes 14, 17, 18). These incubations were at 30 °C for 20 min (lanes 1–13) or for 30 min (lanes 14–18). PPase 2A treatment (0.5 µl of stock, see legend to Fig. 1) was for 30 min at 30 °C after the CK-II pretreatment. After the pretreatments, double-stranded ³²P-labelled oligonucleotides (1 × MRE) were added and further incubated for 20 min at 25 °C. F, free probe; B, bound probe.

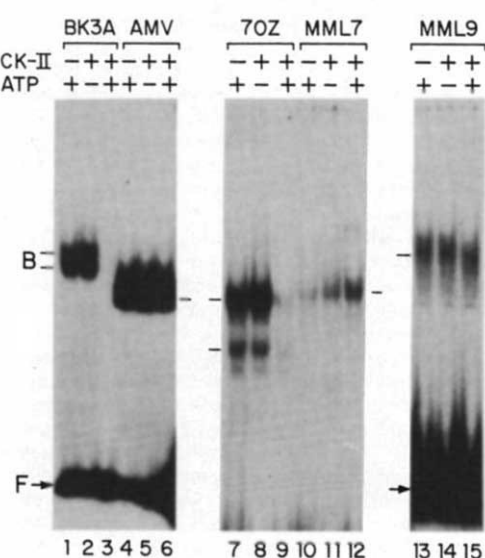
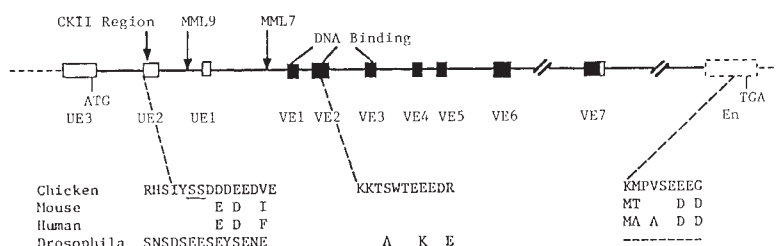


FIG. 5 Loss of the N-terminal CK-II site results in DNA-binding independent of CK-II phosphorylation. Myb was partially purified as described in the legend to Fig. 3 from BK3A cells (lanes 1–3), the AMV-transformed chicken myeloblastic cell line BM2/C3 (lanes 4–6), the mouse pre-B cell lymphoma 70Z (lanes 7–9), the Mo-MuLV-induced myeloid leukaemias MML7 (lanes 10–12) and MML9 (lanes 13–15). Pretreatments with CK-II (72 ng) and with ATP (0.5 mM) were as indicated. After the 20-min preincubation with CK-II ³²P-labelled 1 × MRE (lanes 1–6) or 2 × MRE (lanes 7–15) were added.

FIG. 6 Structure of murine *c-myb* gene. Boxes indicate protein-coding exons in murine *c-myb* separated by introns (not to scale). Other exons probably exist at both ends. The ATG indicates the probable translational start site for p75^{c-myb} whereas the termination codon is located in a so-far poorly defined exon-like region (En). The black boxes indicate the exons transduced (VE1-VE7) by avian myeloblastosis virus (AMV) v-*myb*; the E26 v-*myb* is truncated at the 3' end of exon VE6, and its 5' truncation occurs just downstream of the AMV 5' truncation. The vertical arrows between exons UE2 and VE1 (U, upstream exons of the virally transduced exons) indicate the approximate positions of MuLV integration in the MML7 and MML9 myeloid leukaemia cell lines. Also shown is the region of CK-II phosphorylation mapped in this study. The corresponding amino-acid sequence (single-letter code) in UE2 is indicated with the two Ser phosphorylation sites underlined. The corresponding sequences in chicken, human and *Drosophila myb* are listed^{20,53-55}. The other potential CK-II consensus sequences are also shown in VE2 and En. Our mapping data indicate that these are not phosphorylated.



the specific binding (Fig. 5, lanes 10–15). Therefore the smaller truncations at the N-terminus that delete the CK-II phosphorylation site allow Myb to bind to DNA in the presence of active CK-II.

Discussion

Our results demonstrate that specific interaction with the MRE is abolished when c-Myb is phosphorylated at an authentic *in vivo* site. There is increasing evidence that Myb functions through the MRE as a transcriptional activator^{22,23,25}. Binding to the MRE seems to be critical for the stimulation of expression of a cloned myeloid-specific Myb-regulated gene and several reporter gene constructs²⁵. We expect that CK-II phosphorylation acts to negatively regulate c-Myb-specific transcription from promoters that require binding to the MRE. Given that the phosphorylation site is ~50 residues from the beginning of the DNA-binding domain, phosphorylation could effect long-range structural alterations⁴⁷, or promote folding of the N-terminal region so that the DNA-binding domain is 'masked'. The negative regulation of DNA-binding is unlikely to be through inhibition of dimer formation, because Myb binds DNA as a monomer⁴⁸ and does not seem to require other proteins for its interaction with DNA²¹. Recently several MREs found in the promoter region of a myeloid-specific Myb-responsive gene have been shown to differ in their apparent affinity for Myb²⁵. CK-II phosphorylation completely inhibits Myb binding to the MREs with low binding affinities but is not very efficient at inhibiting Myb binding to the MRE with the highest binding affinity (B.L., unpublished results), indicating that phosphorylation might not totally inhibit all Myb DNA-binding, but rather shift binding activity towards higher-affinity sites.

Our results indicate that both phosphorylated and unphosphorylated populations of c-Myb are present in the cell. The stoichiometry of phosphorylation, and therefore the size of the c-Myb pool capable of binding MREs at any given time, would be expected to be dependent on the balance between CK-II and phosphatase activities. Phosphorylation by CK-II is increased³³⁻³⁶ as well as decreased by certain growth factors³⁷ in some cell lines. We cannot yet directly relate modulation of CK-II activity to specific Myb-regulated responses, because effects of haematopoietic growth factors on CK-II have not been reported. Nonetheless it is conceivable that haematopoietic colony-stimulating factors, which couple proliferation to differentiation of progenitor cells⁴⁹, increase CK-II activity, resulting in phosphorylation of c-Myb and downregulation of expression of stage-specific genes. Protein kinase A (but not several other kinases) can phosphorylate Myb relatively weakly near the N terminus, resulting in partial inhibition of binding to the MRE (B.L., unpublished results). Although we do not know whether this corresponds to an *in vivo* phosphorylation site, it raises the possibility that several signal pathways converge to modulate Myb DNA-binding activity. In this regard it is intriguing that nearly all the genetic rearrangements associated with oncogenic activation of *c-myb* result in deletion of the

target region for phosphorylation. These include the AMV and E26 v-Myb avian retrovirus transductants, and the truncated c-Myb proteins generated by retroviral insertion in murine myeloid leukaemias and chicken bursal lymphomas (refs 14–16 W. Hayward and E. Humphries, personal communication). But the fact that two *c-myb* rearrangements in myeloid tumours involve only 3' truncations⁵⁶ indicates that there are other ways in which *myb* can be oncogenically activated. Although loss of the CK-II phosphorylation site could uncouple c-Myb DNA binding and the effects on specific gene transcription by Myb from its normal physiological regulation, other mutations could influence the specificity or rate of transcription. Therefore loss of the CK-II phosphorylation site, 3' truncations, and constitutive *myb* expression, could act synergistically to deregulate c-Myb function. These ideas can be tested by determining the effects of point mutations in the phosphorylation site on the transcriptional and biological activities of c-Myb. □

Received 8 November 1989; accepted 13 February 1990.

- Klempner, K.-H., Symonds, G., Evan, G. & Bishop, J. M. *Cell* **37**, 537–547 (1984).
- Boyle, W. J., Lipsick, J. S. & Baluda, M. A. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4685–4689 (1986).
- Klempner, K.-H., Bonifer, C. & Sippel, A. E. *EMBO J.* **5**, 1903–1911 (1986).
- Boyle, W. J., Lampert, M. A., Li, A. C. & Baluda, M. A. *Molec. cell. Biol.* **5**, 3017–3023 (1985).
- Bading, H., Beutler, C., & Moelling, K. *Oncogene* **4**, 33–38 (1989).
- Clarke, M. F., Kukowska-Latallo, J. F., Westin, E., Smith, M. & Prochownik, E. V. *Molec. cell. Biol.* **8**, 4685–4689 (1988).
- Todokoro, K. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8900–8904 (1988).
- McMahon, J., Howe, K. M. & Watson, R. J. *Oncogene* **3**, 717–720 (1988).
- Duprey, S. P. & Boettiger, D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6937–6941 (1985).
- Graf, T. & Beug, H. *BBA Rev. Cancer* **516**, 269–299 (1978).
- Beug, H., Blundell, P. A. & Graf, T. *Genes Dev.* **1**, 277–286 (1987).
- Durban, E. M. & Boettiger, D. *J. Virol.* **37**, 488–492 (1981).
- Klempner, K.-H. et al. *Cell* **33**, 345–355 (1983).
- Shen-Ong, G. L. C., Potter, M., Mushinski, J. F., Lavi, S. & Reddy, E. P. *Science* **226**, 1077–1080 (1984).
- Kanter, M. R., Smith, R. E. & Hayward, W. S. *J. Virol.* **62**, 1423–1432 (1988).
- Pizer, E. & Humphries, E. H. *J. Virol.* **63**, 1630–1640 (1989).
- Gonda, T. J., Cory, S., Sobieszcuk, P., Holtzman, D. & Adams, J. J. *J. Virol.* **61**, 2754–2763 (1987).
- Ibanez, C. E. & Lipsick, J. S. *J. Virol.* **62**, 1981–1988 (1988).
- Klempner, K.-H. & Sippel, A. E. *EMBO J.* **6**, 2719–2725 (1987).
- Peters, C. W., Sippel, A. E., Vingron, M. & Klempner, K.-H. *EMBO J.* **6**, 1085–1090 (1987).
- Biedenkapp, H., Borgmeyer, U., Sippel, A. E. & Klempner, K.-H. *Nature* **335**, 835–837 (1988).
- Weston, K. & Bishop, J. M. *Cell* **58**, 85–93 (1989).
- Klempner, K.-H., Arnold, H. & Biedenkapp, H. *Genes Dev.* **3**, 1582–1589 (1989).
- Sakura, H. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5758–5762 (1989).
- Ness, S. A., Marknell, A. & Graf, T. *Cell* **59**, 1115–1125 (1989).
- Raychaudhuri, P., Bagchi, S. & Nevins, J. R. *Genes Dev.* **3**, 620–627 (1989).
- Yamamoto, K. K., Gonzalez, G. A., Biggs III, W. A. & Montminy, M. R. *Nature* **334**, 494–498 (1988).
- Cyert, M. S. & Thorne, J. *Cell* **57**, 891–893 (1989).
- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. *A. Rev. Biochem.* **56**, 567–613 (1987).
- Hunter, T. *Cell* **50**, 823–829 (1987).
- Kuenzel, E. A., Mulligan, J. A., Sommercorn, J. & Krebs, E. G. *J. Biol. Chem.* **262**, 9136–9140 (1987).
- Krebs, E. G. et al. *Cold Spring Harb. Symp. quant. Biol.* **53**, 77–84 (1988).
- Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8834–8838 (1987).
- Klarlund, J. K. & Czech, M. P. *J. Biol. Chem.* **263**, 15872–15875 (1988).
- Carroll, D. & Marshak, D. R. *J. Biol. Chem.* **264**, 7345–7348 (1989).
- Ackerman, P. & Osheroff, N. *J. Biol. Chem.* **264**, 11958–11965 (1989).
- Corvera, S., Roach, P. J., DePadi-Roach, A. A. & Czech, M. P. *J. Biol. Chem.* **263**, 3116–3122 (1988).
- Lüscher, B., Kuenzel, E. A., Krebs, E. G. & Eisenman, R. N. *EMBO J.* **8**, 1111–1119 (1989).
- Firzlaff, J. M., Galloway, D. A., Eisenman, R. N. & Lüscher, B. *New Biologist* **1**, 44–53 (1989).
- Lüscher, B. & Eisenman, R. N. *Molec. cell. Biol.* **8**, 2504–2512 (1988).
- Meyer, H. E., Hoffman-Pororske, E., Korte, H. & Heilmeyer, L. M. G. *J. FEBS Lett.* **204**, 61–66 (1986).
- Holmes, C. F. B. *FEBS Lett.* **215**, 21–24 (1987).
- Revzin, A. *Bio Techniques* **7**, 346–355 (1989).
- Lassar, A. B. et al. *Cell* **58**, 823–831 (1989).
- Shen-Ong, G. L. C. & Wolff, L. J. *J. Virol.* **61**, 3721–3725 (1987).
- Shen-Ong, G. L. C., Lüscher, B. & Eisenman, R. N. *Molec. cell. Biol.* **9**, 5456–5463 (1989).

47. Sprang, S. R. *et al.* *Nature* **336**, 215–221 (1988).
 48. Howe, K. A., Reakes, C. F. L. & Watson, R. J. *EMBO J.* **9**, 161–169 (1990).
 49. Metcalf, D. *The Molecular Control of Blood Cells* (Harvard University Press, 1988).
 50. Kazlauskas, A. & Cooper, J. A. *Cell* **58**, 1121–1133 (1988).
 51. Evan, G., Lewis, G. K. & Bishop, J. M. *Molec. cell. Biol.* **4**, 2843–2850 (1989).
 52. Klemmner, K.-H. *et al.* *Cell* **33**, 345–355 (1983).
 53. Gerondakis, S. & Bishop, J. M. *Molec. cell. Biol.* **6**, 3677–3684 (1986).
 54. Gonda, T. J., Gough, N. M., Dunn, A. R. & de Blaquiere, J. *EMBO J.* **4**, 2003–2008 (1985).
 55. Majello, B., Kenvon, L. C. & Dalla-Favera, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9636–9640 (1986).

56. Weinstein, Y., Cleveland, J. L., Askew, D. S., Rapp, U. R. & Ihle, J. N. *J. Virol.* **61**, 2339–2343 (1987).

ACKNOWLEDGEMENTS. We dedicate this paper to the late Elizabeth Kuenzel who began this work. We thank B. Boyle, P. Chou, J. Cooper, E. Fischer, M. Harrylock, F. Kennedy, C. Piening, G. Ramsey, G. Shen-Ong, J. Sommercorn, E. Tolentino, N. Tonks, L. Wolff and H. Zebroski for reagents, cells and advice, E. Humphries, W. Hayward, T. Graf and R. Watson for unpublished data, and J. Cooper and A. Lassar for critically reading the manuscript. This work was supported by a grant, from the National Cancer Institute (NIH) to R.N.E. B.L. was supported by the Swiss NSF, and D.W.L. was supported by the MRC of Canada.

LETTERS TO NATURE

OH-IR sources as precursors to protoplanetary nebulae

M. C. Shepherd*, R. J. Cohen*, M. J. Gaylard†
& M. E. West†

* University of Manchester, Nuffield Radio Astronomy Laboratories,
Jodrell Bank, Macclesfield, Cheshire SK11 9DL, UK

† Hartebeesthoek Radio Astronomy Observatory, PO Box 443,
Krugersdorp 1740, South Africa

It has long been suspected¹ that OH-IR sources, highly evolved red giant stars that have built up massive, cool gaseous envelopes through heavy mass loss, are precursors to planetary nebulae. The two kinds of object share a similar galactic distribution, and their circumstellar envelopes have comparable masses and expansion velocities^{2,3}. Recently, several hybrid objects have been found with the far infrared and OH maser emission characteristic of the OH-IR sources, but also with radio continuum emission from a central H II region^{4–6}. There is strong circumstantial evidence that these objects, of which the prototype is Vy2-2 (refs 4, 7), seem to be in a transitional state, but their precise evolutionary status remains unclear. Here we present radio-interferometer maps, obtained with the MERLIN network, of OH maser emission at 1,612 MHz from Vy2-2 and OH0.9+1.3, another of the hybrid sources. Our maps reveal shell structure in the masers, which we take to be conclusive evidence of an evolutionary link between OH-IR sources and planetary nebulae.

There are many observational difficulties in tracing the evolution of OH-IR sources. The high visual extinction of their dense envelopes means that the central star cannot be studied directly: only radio and infrared data on the envelope are generally available. Several possible transition objects have been reported which show OH emission at 1,612 MHz and radio continuum emission^{4–6}, but these properties do not identify them unambiguously as young planetary nebulae. In one of the best-studied cases it was not possible even to distinguish whether the obscured source was a protoplanetary nebula, a very young (pre-main-sequence) star with a compact H II region, or a post-main-sequence object⁶. To clarify the nature of such sources, high-resolution observations are needed to characterize the structure of the circumstellar envelope.

Previous measurements of the hybrid sources Vy2-2 and OH0.9+1.3 made using the Very Large Array (VLA) and MERLIN have shown that the 1,612-MHz OH maser regions are only a few tenths of an arcsecond in angular extent^{7–9}. Therefore these two sources were among the first to be observed using the MERLIN network extended to Cambridge¹⁰. An 18-m dish from the Cambridge One-Mile Telescope was made available for MERLIN spectral line observations in April–May 1988. This was used together with the 76-m Lovell telescope at Jodrell Bank and 26-m out-station telescopes at Knockin and Defford to form an interferometer array with a longest baseline of 234 km and a minimum lobe-spacing of 0.16 arcsec at 1,612 MHz. The observational methods will be described in more detail elsewhere. Maps of both sources made using the normal self-calibration procedures are shown in Figs 1 and 2. The new measurements resolve the masers for the first time. The surface bright-

ness, expressed as brightness temperature, is $\sim 10^6$ – 10^8 K, which is typical of OH-IR sources and is orders of magnitude less than the values for OH masers associated with young stars and compact H II regions¹¹. Both sources show evidence of the shell structure characteristic of OH-IR sources^{12,13}. This is clearest in the weak redshifted emission from OH0.9+1.3 at velocities of around -93 km s⁻¹. The channel maps show successive slices through the shell at constant radial velocity. OH0.9+1.3 lies near the Galactic Centre^{9,14}, and is the first OH-IR source in this region to be resolved.

The OH shells of OH-IR sources are usually well approximated by a thin shell of constant radius and constant expansion velocity^{12,13}. The results of fitting such a model to our data are shown in Fig. 3, which shows angular radius of the emission region as a function of velocity. Our fitting procedure uses the second moment of each channel map about the assumed stellar

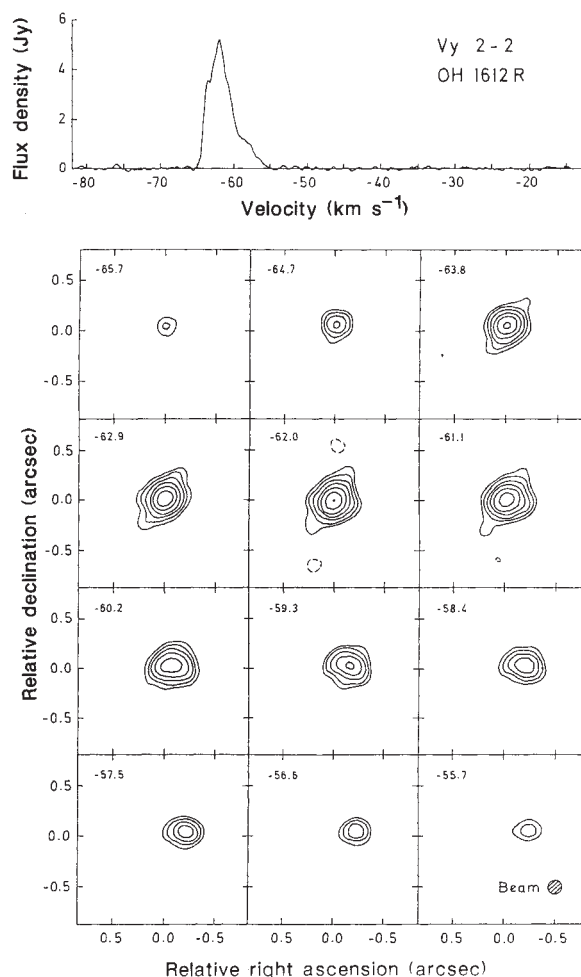


FIG. 1 Maps of OH 1,612-MHz emission from Vy2-2 at selected velocities. Velocities are indicated in the top left-hand corner of each map. The velocity resolution is 1.3 km s⁻¹ and the restoring beam used is 0.15 arcsec in half-power width. Contours are plotted at -0.05 (dashed), 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 Jy per beam. The total power spectrum is shown above for reference.

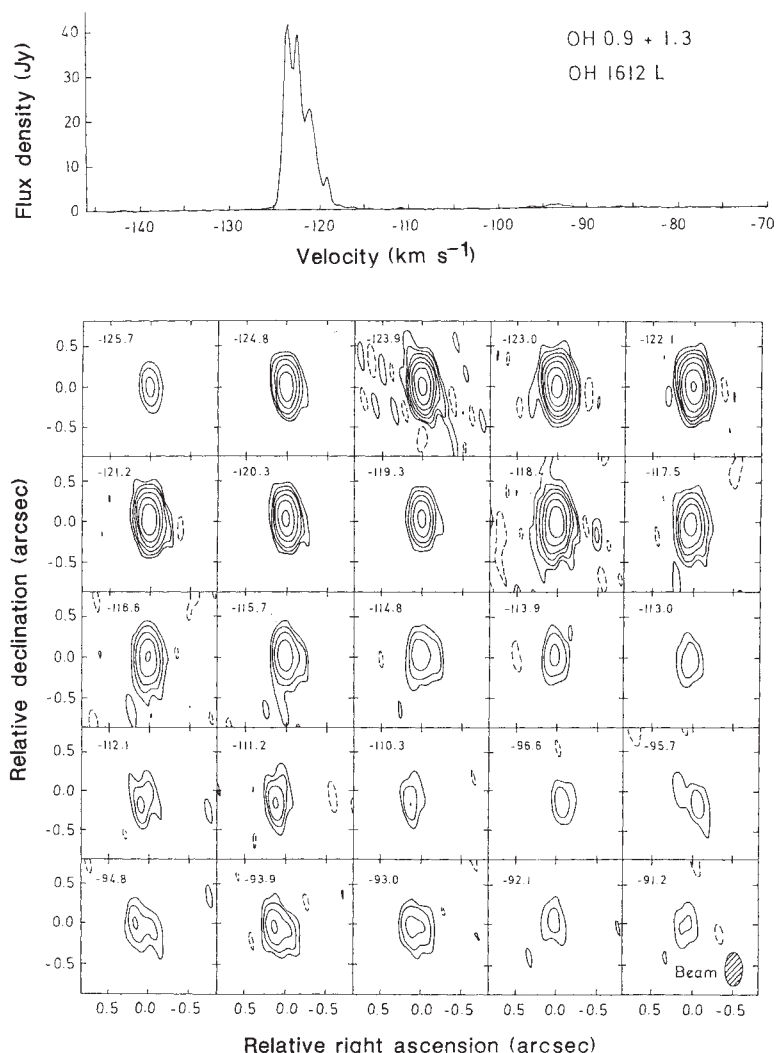


FIG. 2 Maps of OH 1,612-MHz emission from OH0.9+1.3 at selected velocities. Velocities are indicated in the top left-hand corner of each map. The velocity resolution is 1.3 km s^{-1} and the restoring beam is $0.2 \times 0.4 \text{ arcsec}$. Contours are plotted at -0.05 (dashed), $0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8$ and 25.6 Jy per beam except in the maps of strongest emission (-119.3 to -125.7 km s^{-1}), where contours below 0.4 were omitted and a dashed contour at -0.4 was added. The total power spectrum is shown above for reference.

position, and incorporates corrections for the beam size and the size of individual maser clumps. The thin-shell model provides an excellent fit to the redshifted emission from OH0.9+1.3, with a shell of radius $0.24 \pm 0.03 \text{ arcsec}$, an expansion velocity of $15.2 \pm 0.5 \text{ km s}^{-1}$ and a central velocity of $-107.0 \pm 0.5 \text{ km s}^{-1}$. The velocity parameters agree well with those determined from recent measurements of CO in the outer molecular envelope¹⁴. Absolute position measurements made with the VLA confirm that the shell centre derived here coincides with the position of the radio continuum source to within the errors of measurement, $\pm 0.3 \text{ arcsec}$ ^{8,9}. The redshifted emission originates on the far side of the expanding shell, and so will be partly occulted by the optically thick H II region^{4,7}. This may help to explain the relative weakness of the redshifted emission. We predict that high-resolution Very-Long-Baseline Interferometry (VLBI) measurements will show a central hole in the redshifted cap, delineating the compact H II region. Not all the emission from OH0.9+1.3 follows the thin-shell model. The powerful blue-shifted emission near -120 km s^{-1} comes instead from a more compact region nearer the line of sight to the star, and was therefore excluded from the least-squares fit. This powerful emission may be associated with the disruption of the inner parts of the molecular envelope either by stellar ultraviolet radiation or by the onset of a fast stellar wind. It is possible that weaker shell-type emission is also present but is being masked.

Vy2-2 has a far less complete OH shell, with only a fragment remaining to the west of the compact H II region⁷. The regular shift in position with velocity is again consistent with an

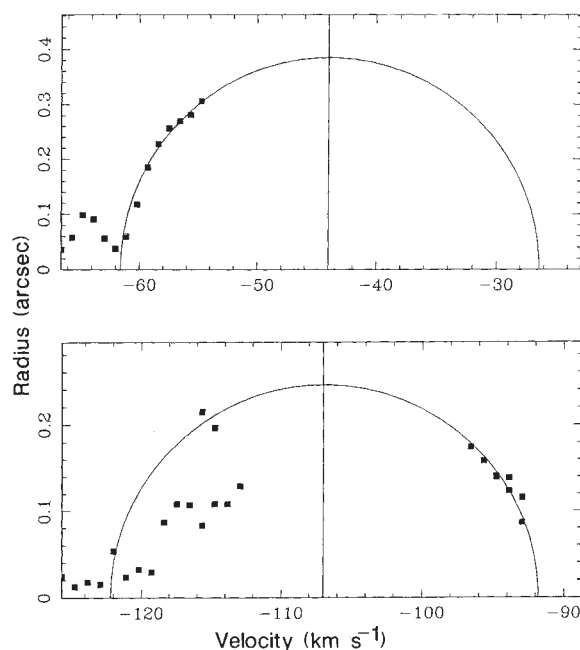
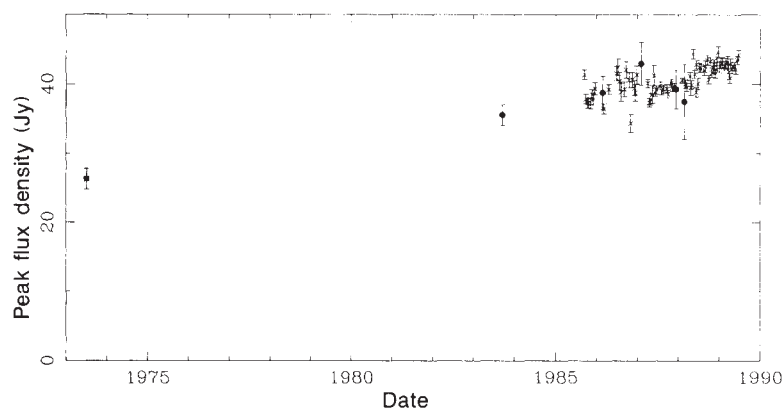


FIG. 3 Angular radius of the maser emission regions as a function of radial velocity for Vy2-2 (upper) and OH0.9+1.3 (lower). The dashed lines show the best-fitting thin-shell model in each case. The model parameters are given in the text.

FIG. 4 The peak flux density of the OH 1,612-MHz emission from OH0.9+1.3 plotted as a function of time. Crosses show Hartebeesthoek measurements, circles show Jodrell Bank measurements, and the square is the discovery measurement taken from ref. 21.



expanding-shell model, as shown in Fig. 3. Because the data do not properly constrain a least-squares fit of all parameters, we adopted the stellar velocity of -44 km s^{-1} found from CO measurements¹⁵. This leads to a shell radius of 0.39 ± 0.05 arcsec, an expansion velocity of 18 km s^{-1} (which agrees with the CO data) and a stellar position that agrees with the position of the compact H II region to within the combined errors of ± 0.2 arcsec.

It is well established that the OH shell radii of OH-IR sources increase systematically with mass loss rate^{12,16,17}. This comes about because the OH is produced when H_2O molecules in the outflowing gas are photodissociated by the external ultraviolet radiation. For a spherically symmetric outflow, the OH photodissociation radius is then determined by the mass loss rate and the strength of the ambient ultraviolet radiation field. By a happy coincidence, physical conditions at this photodissociation radius are also right for pumping the OH 1,612-MHz maser¹⁸. Mass loss rates for Vy2-2 and OH0.9+1.3 have been determined as $2.4 \times 10^{-6} M_{\odot} \text{ yr}^{-1}$ and $3.0 \times 10^{-5} M_{\odot} \text{ yr}^{-1}$ for assumed distances of 1 and 8 kpc respectively^{14,15}. These imply OH photodissociation radii of $6.3 \times 10^{13} \text{ m}$ and $3.9 \times 10^{14} \text{ m}$ for an average interstellar ultraviolet flux, which agree well with the values of $5.8 \times 10^{13} \text{ m}$ and $2.9 \times 10^{14} \text{ m}$ derived from our measurements. Thus the OH shells in these two sources are indeed fossil remnants of the OH-IR phase, having the brightness temperatures and shell radii characteristic of OH-IR sources.

Clearly we have been fortunate to catch these two objects in the transition phase: the expansion timescale of the compact H II regions is only 300 years. In fact, the molecular envelopes could evolve even more rapidly, particularly if there is a fast wind, as suggested by recent observations of Herbig-Haro objects and high-velocity outflows in other protoplanetary nebula candidates^{19,20}. Strong changes in the OH maser emission could then occur as the inner parts of the envelope are disrupted. It is possible that such disruption is now being observed in OH0.9+1.3 in the powerful masers at high negative velocity. Single-telescope monitoring has revealed a steady increase in the negative-velocity peak (Fig. 4). The flux density of the emission seems to have increased at the rate of 1 Jy yr^{-1} since the source was discovered in 1973²¹. Such behaviour is unprecedented for an OH-IR source. The implied age of the feature is about 40 yr. It may be possible to resolve the structure of these masers using VLBI. Measurements of the temporal evolution of this remarkable source could be crucial to understanding the evolutionary path from OH-IR sources to planetary nebulae. □

9. Zijlstra, A.A. *et al. Astr. Astrophys.* **217**, 157-178 (1989).
10. Thomasson, P. Q. *J. R. astr. Soc.* **27**, 413-431 (1986).
11. Fouquet, J. E. & Reid, M. J. *Astr. J.* **87**, 691-694 (1982).
12. Bowers, P. F., Johnston, K. J. & Spencer, J. H. *Astrophys. J.* **274**, 733-754 (1983).
13. Diamond, P. J., Norris, R. P., Rowland, P. R., Booth, R. S. & Nyman, L.-A. *Mon. Not. R. astr. Soc.* **212**, 1-21 (1985).
14. Mauersberger, R., Henkel, C., Wilson, T. L. & Olano, C. A. *Astr. Astrophys.* **206**, L34-L36 (1988).
15. Knapp, G. R. & Morris, M. *Astrophys. J.* **292**, 640-669 (1985).
16. Huggins, P. J. & Glassgold, A. E. *Astr. J.* **87**, 1828-1835 (1982).
17. Netzer, N. & Knapp, G. R. *Astrophys. J.* **323**, 734-748 (1987).
18. Cohen, R. J. *Rep. Prog. Phys.* **52**, 881-943 (1989).
19. Reipurth, B. *Nature* **325**, 787-790 (1987).
20. te Lintel Hekkert, P., Habing, H. J., Caswell, J. L., Norris, R. P. & Haynes, R. F. *Astr. Astrophys.* **202**, L19-L22 (1988).
21. Kerr, F. J. & Bowers, P. F. *Astr. Astrophys.* **36**, 225-229 (1974).

Positioning single atoms with a scanning tunnelling microscope

D. M. Eigler & E. K. Schweizer*

IBM Research Division, Almaden Research Center, 650 Harry Rd, San Jose, California 95120, USA

SINCE its invention in the early 1980s by Binnig and Rohrer^{1,2}, the scanning tunnelling microscope (STM) has provided images of surfaces and adsorbed atoms and molecules with unprecedented resolution. The STM has also been used to modify surfaces, for example by locally pinning molecules to a surface³ and by transfer of an atom from the STM tip to the surface⁴. Here we report the use of the STM at low temperatures (4 K) to position individual xenon atoms on a single-crystal nickel surface with atomic precision. This capacity has allowed us to fabricate rudimentary structures of our own design, atom by atom. The processes we describe are in principle applicable to molecules also. In view of the device-like characteristics reported for single atoms on surfaces^{5,6}, the possibilities for perhaps the ultimate in device miniaturization are evident.

The tip of an STM always exerts a finite force on an adsorbate atom. This force contains both Van der Waals and electrostatic contributions. By adjusting the position and the voltage of the tip we may tune both the magnitude and direction of this force. This, taken together with the fact that it generally requires less force to move an atom along a surface than to pull it away from the surface, makes it possible to set these parameters such that the STM tip can pull an atom across a surface while the atom remains bound to the surface. Our decision to study xenon on nickel (110) was dictated by the requirement that the corrugations in the surface potential be sufficiently large for the xenon atoms to be imaged without inadvertently moving them, yet sufficiently small that, when desired, enough lateral force could be exerted to move xenon atoms across the surface.

The experiments were performed using an STM contained in an ultra-high-vacuum system and cooled to 4 K. The entire

Received 9 November 1989; accepted 16 February 1990.

1. Elitzur, M., Goldreich, P. & Scoville, N. *Astrophys. J.* **205**, 384-396 (1976).
2. Habing, H. J., te Lintel Hekkert, P. & van der Veen, W. E. C. J. in *Planetary Nebulae* (ed. Torres-Peimbert, S.) 359-380 (Kluwer, Dordrecht, 1989).
3. Pottasch, S. R. *Planetary Nebulae* (Reidel, Dordrecht, 1984).
4. Davis, L. E., Seaquist, E. R. & Purton, C. R. *Astrophys. J.* **230**, 434-441 (1979).
5. Pottasch, S. R., Bignelli, C. & Zijlstra, A. *Astr. Astrophys.* **177**, L49-L52 (1987).
6. Zijlstra, A. A. & Pottasch, S. R. *Astr. Astrophys.* **196**, L9-L12 (1988).
7. Seaquist, E. R. & Davis, L. E. *Astrophys. J.* **274**, 659-665 (1983).
8. Winnberg, A., Terzides, C. & Matthews, H. E. *Astr. J.* **86**, 410-417 (1981).

* Permanent address: Fritz-Haber-Institut, Faradayweg 4-6, D-7000 Berlin 33, FRG.

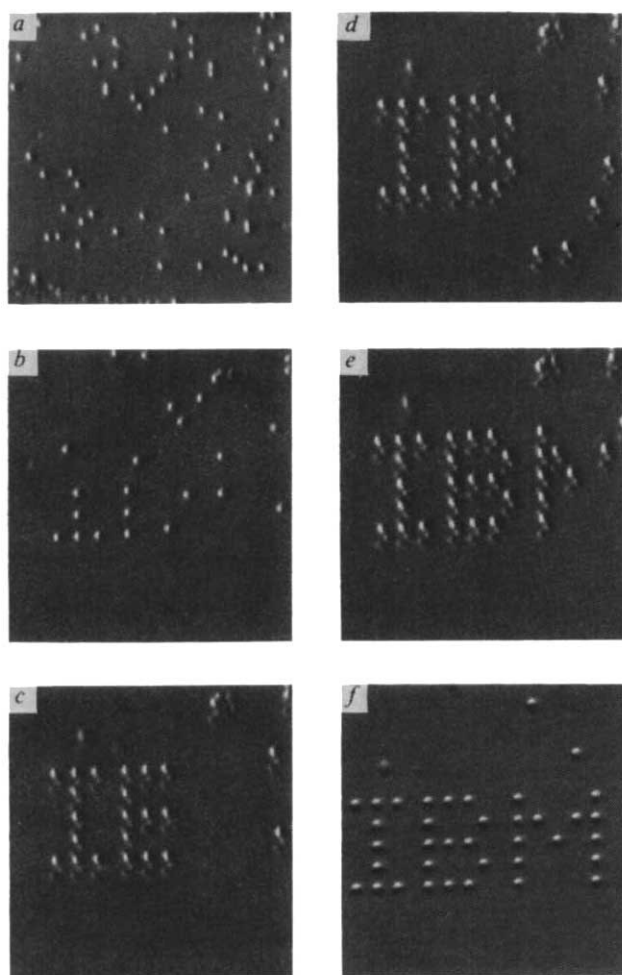


FIG. 1 A sequence of STM images taken during the construction of a patterned array of xenon atoms on a nickel (110) surface. Grey scale is assigned according to the slope of the surface. The atomic structure of the nickel surface is not resolved. The $\langle 1\bar{1}0 \rangle$ direction runs vertically. *a*, The surface after xenon dosing. *b-f*, Various stages during the construction. Each letter is 50 Å from top to bottom.

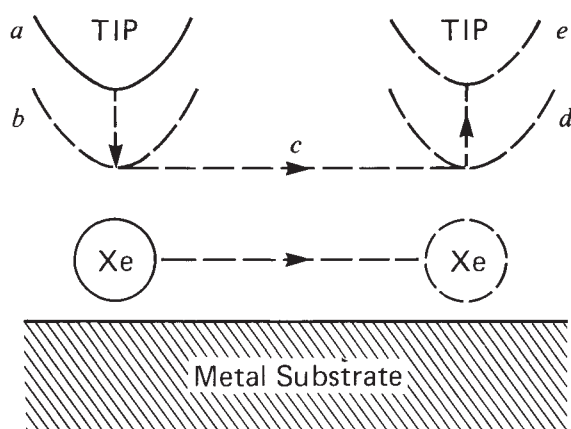


FIG. 2 A schematic illustration of the process for sliding an atom across a surface. The atom is located and the tip is placed directly over it (*a*). The tip is lowered to position (*b*), where the atom-tip attractive force is sufficient to keep the atom located beneath the tip when the tip is subsequently moved across the surface (*c*) to the desired destination (*d*). Finally, the tip is withdrawn to a position (*e*) where the atom-tip interaction is negligible, leaving the atom bound to the surface at a new location.

chamber housing the microscope was cooled to 4 K, which so reduced the contamination rate of the sample surface through adsorption of residual gases that no measurable contamination occurred over weeks. The stability of the sample and of the microscope due to the low temperature are such that one may perform experiments on a single atom for days at a time. This stability proved to be an operational necessity for performing these experiments. This notwithstanding, it is important to realize that the process that we describe for sliding atoms on a surface is fundamentally temperature independent.

The nickel sample was processed by cycles of argon-ion sputtering, annealing in a partial pressure of oxygen to remove surface carbon, and flash annealing. After cooling to 4 K the sample was imaged with the STM and found to be of acceptable quality. Figure 1*a* is an image of the surface taken under constant-current scanning conditions after dosing with xenon to the desired coverage. This image was obtained with a tip bias voltage of 0.010 V relative to the sample, and a tunnel current of 10^{-9} A. Each xenon atom appears as a 1.6-Å-high bump on the surface, apparently at random locations. At this gap impedance the interaction of the xenon with the tip is sufficiently weak to leave the xenon essentially unperturbed during the imaging process.

To move an atom we follow the sequence of steps depicted in Fig. 2. We begin by operating the microscope in the non-perturbative imaging mode described above to locate the atom to be moved and to target its destination. We stop scanning and place the tip directly above the atom to be moved (*a*). We then increase the tip-atom interaction by lowering the tip toward the atom (*b*); this is achieved by changing the required tunnel current to a higher value, typically in the range $1-6 \times 10^{-8}$ A, which causes the tip to move towards the atom until the new tunnel current is reached. We then move the tip under closed-loop conditions across the surface, (*c*), at a speed of 4 Å per second to the desired destination (*d*), dragging the xenon atom with it. The tip is then withdrawn (*e*) by reducing the tunnel current to the value used for imaging. This effectively terminates the attraction between the xenon and the tip, leaving the xenon bound to the surface at the desired location.

Figure 1 is a sequence of images taken during our first construction of a patterned array of atoms, and demonstrates our ability to position atoms with atomic precision. The exact periodicity of the xenon spacing is derived from the crystalline structure of the underlying nickel surface (which is not resolved in Fig. 1). The nickel (110) surface has an unreconstructed rectangular unit cell and is oriented such that the short dimension of the rectangular surface unit cell runs vertically in the image. The xenon atoms are spaced on a rectangular grid which is four nickel unit cells long horizontally and five unit cells long vertically, corresponding to 14×12.5 Å.

Although the STM tip is made from tungsten wire, the true chemical identity and the structure of the outermost atoms of the tip are not known to us. We find that for any given tip and bias voltage, there is a threshold height below which the tip must be located to be able to move xenon atoms parallel to the rows of nickel atoms, and a lower threshold height for movement perpendicular to the rows of nickel atoms. This is consistent with a simple model wherein the xenon interaction with the metal surface is approximated by pairwise interactions with the individual nickel atoms. Simple investigations showed that the magnitude or sign of the applied voltage had no significant effect on the threshold tip height. This suggests that the dominant force between the tip and the xenon atom is due to the Van der Waals interaction, but this tentative conclusion requires further investigation.

In Fig. 3 we show a sequence of images demonstrating how a simple structure, the linear multimer, may be fabricated using this process. First we slide xenon atoms into a chosen row of nickel atoms. We next slide a xenon atom along the row to a position where it will bind with a neighbouring xenon to form

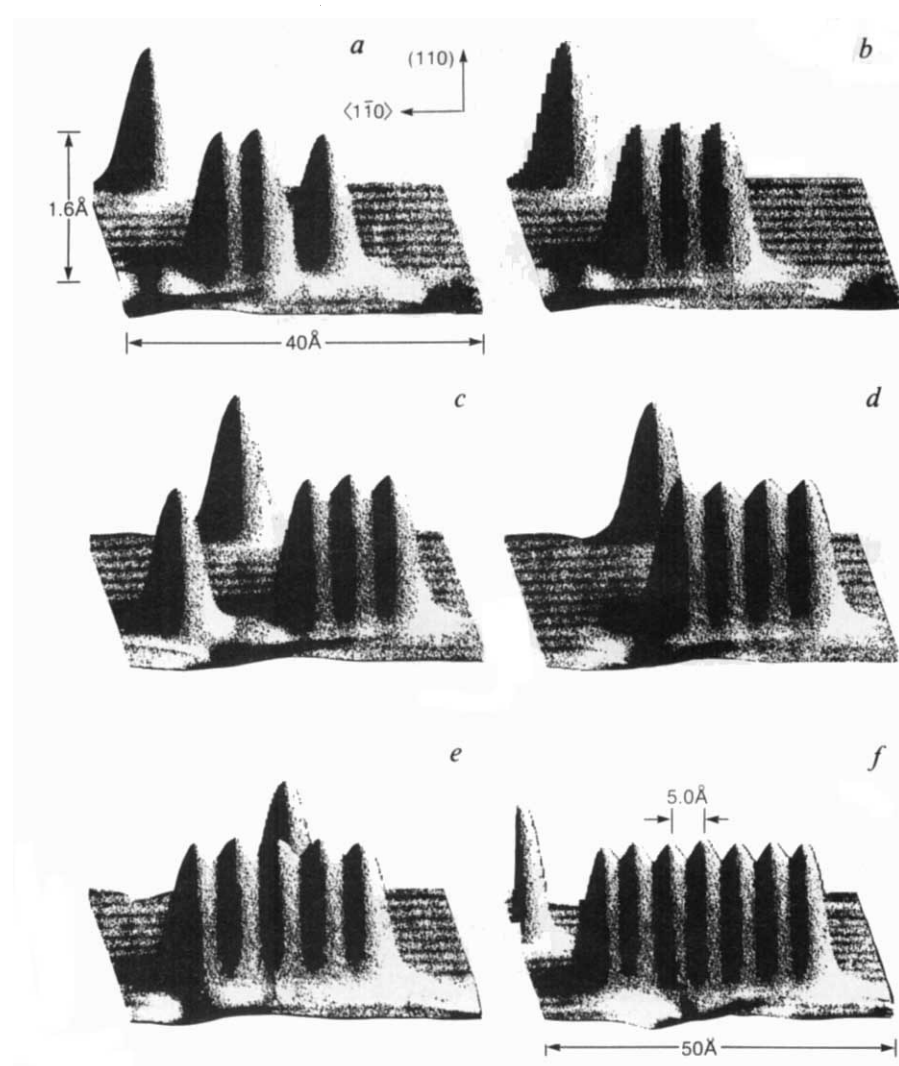


FIG. 3 Various stages in the construction of a linear chain of xenon atoms on the nickel (110) surface. The individual xenon atoms appear as 1.6-Å-high protrusions in these images. The rows of nickel atoms are visible as alternating light and dark stripes. Several point defects are visible in the nickel surface. *a*, The assembled xenon dimer. To the right of the dimer, a xenon atom has been moved into position for forming a xenon trimer. *b*, Formation of the xenon linear trimer. *c*–*f*, Various stages in construction of the linear heptamer, a process that can be completed in an hour. The xenon atoms are 5 Å apart, occupying every other unit cell of the nickel surface.

a dimer. We repeat the process, forming a linear trimer, then a linear tetramer, and so on. From these images we find that the linear xenon chain along the $\langle 110 \rangle$ direction of the nickel (110) surface is stable, the xenon atoms occupying every other surface unit cell along a row of nickel atoms. Attempts to pack the xenon atoms closer were unsuccessful. We find the xenon–xenon spacing along the row to be uniform (excluding end effects) to within 0.2 Å.

We anticipate that there will be a limiting class of adsorbed atoms and molecules that may be positioned by this method. Many new avenues of investigation are open to us. It should be possible to assemble or modify certain molecules in this way. We can build novel structures that would otherwise be unobtainable. This will allow a new class of surface studies that use the STM both to fabricate overlayer structures and to probe their properties. The prospect of atomic-scale logic circuits and other devices is a little less remote. □

A tri-platinum complex containing a coordinatively naked platinum atom

T. Tanase*, Y. Kudo*, M. Ohno*, K. Kobayashi† & Y. Yamamoto*

* Department of Chemistry, Faculty of Science, Toho University, Funabashi-shi, Chiba 274, Japan

† Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351, Japan

POLYNUCLEAR clusters of platinum and palladium containing metal–metal bonds may serve as models of the surface of heterogeneous catalysts, and their reactions may mimic those on such surfaces^{1,2}. Ligand-free metal clusters can be created in an ultradispersed condensed phase³, but they are difficult to isolate and characterize because of their high reactivities. Here we report the preparation of a tri-platinum diphosphine complex that contains a coordinatively ‘naked’ platinum atom. The metal cluster in this complex is electron-deficient relative to other tri-platinum complexes. Bonding of ligands to the naked platinum could reflect that of chemisorbed species on catalytic platinum surfaces.

The potentiostatic electrolysis of $[\text{Pt}(\text{diphos})(\text{RNC})_2](\text{PF}_6)_2$

Received 17 January; accepted 9 March 1990.

1. Binnig, G., Rohrer, H., Gerber, Ch. & Weibel, E. *Appl. Phys. Lett.* **40**, 178–180 (1982).
2. Binnig, G., Rohrer, H., Gerber, Ch. & Weibel, E. *Phys. Rev. Lett.* **49**, 57–61 (1982).
3. Foster, J. S., Frommer, J. E. & Arnett, P. C. *Nature* **331**, 324–326 (1988).
4. Becker, R. S., Golovchenko, J. A. & Swartzentruber, B. S. *Nature* **325**, 419–421 (1987).
5. Lyo, I.-W. & Avouris, P. *Science* **245**, 1369–1371 (1989).
6. Bedrossian, P., Chen, D. M., Mortensen, K. & Golovchenko, J. A. *Nature* **342**, 258–260 (1989).

ACKNOWLEDGEMENTS. This work would not have occurred were it not for the patient and visionary management of the IBM Research Division.

(diphos = dppe (1,2-bis(diphenylphosphino)ethane), R = 2,6-(CH₃)₂C₆H₃ (compound 1A); diphos = dppp (1,3-bis(diphenylphosphino)propane), R = 2,6-(CH₃)₂C₆H₃ (1B); diphos = dppp, R = 2,4,6-(CH₃)₃C₆H₂ (1C)) was carried out in a 0.1M CH₃CN solution containing NaClO₄ as a supporting electrolyte, using a mercury-pool electrode at -1.6 V (relative to saturated calomel electrode, SCE). The electrolysis consumed ~1.0 F and produced a pale yellow compound, formulated as [Pt₂(diphos)₂(RNC)₂](PF₆)₂ (diphos = dppe; R = 2,6-(CH₃)₂C₆H₃ (compound 2A; 22% yield); diphos = dppp, R = 2,6-(CH₃)₂C₆H₃ (2B; 19% yield); diphos = dppp, R = 2,4,6-(CH₃)₃C₆H₂ (2C; 55% yield)). Spectral and analytical studies of compound 2 suggest that it has a binuclear structure without bridging isocyanides. An X-ray crystallographic analysis of 2C showed that the binuclear structure comprises two square planar PtP₂C planes joined by a metal-metal single bond (Pt(1)-Pt(2), bond length 2.653(2) Å) (Fig. 1A)^{4,5}. This bond length is significantly greater than that in Pt₂Cl₂(2,4-*t*-(C₆H₅)₂-6-(CH₃)C₆H₂NC)₄ (compound 3) (Pt-Pt = 2.562 Å)^{6,7}. The dihedral angle between the two coordination planes in 2C is 86°. The two bidentate dppp ligands are chelating, with an average bite angle of 94.1°, and the six-membered chelate rings adopt the stable 'chair' conformation. Similar zero-valent dimeric platinum complexes containing chelating diphosphines have been described by Yoshida *et al.*⁸, but they are nevertheless rare.

When 1.5 F mol⁻¹ was passed through a solution of compound 1A at -1.5 V (relative to SCE), an orange-red complex, formulated as [Pt₃(dppe)₂(2,6-(CH₃)₂C₆H₃NC)₄](PF₆)₂·CH₂Cl₂ (compound 4A), was obtained in 70% yield. The infrared spectrum of 4A showed a peak at 2,115 cm⁻¹, which we assigned to the terminal isocyanide groups. The ¹H NMR spectrum indicated the presence of two kinds of isocyanide ligands (chemical shifts δ = 1.86 and 2.25 for the 2,6-CH₃ protons). From these spectral and analytical data, and by comparison with the

structure of [Pt₃(2,6-(CH₃)₂C₆H₃NC)₆(PPh₃)₂](PF₆)₂·CH₂Cl₂ (compound 5)⁹, we assigned to 4A a trinuclear structure with a linear arrangement of the three Pt atoms:



By contrast, using dppp as the diphosphine ligand, reduction of 1B by 1.5 F mol⁻¹ gave the dimer (2B, 40% yield) and an orange compound formulated as [Pt₃(dppp)₂(2,6-(CH₃)₂C₆H₃NC)₂](PF₆)₂ (compound 4B, 11% yield). The electronic (ultraviolet-visible) spectrum of 4B is very similar to that of 4A, but is shifted to higher energies by ~3 × 10³ cm⁻¹. Preliminary EXAFS (extended X-ray absorption fine structure) spectra for 4A, 4B and 5 were measured at the Photon Factory of National Laboratory for High Energy Physics. The Fourier transforms of these spectra are similar, showing three peaks due to C, P and Pt. These are consistent with the proposed linear tri-platinum structure. Furthermore, the average distances between platinum atoms are almost identical for 4A, 4B and 5. Thus 4B seems to have the same tri-platinum core as is found in 4A and 5. But the elemental analysis and ¹H NMR spectrum (there is a single signal at δ = 2.00 for the 2,6-CH₃ groups) imply that the Pt atoms in 4B are coordinatively unsaturated.

We performed an X-ray crystallographic analysis of 4B to elucidate the unsaturated structure (Fig. 1B)^{4,5}. This shows that the three Pt atoms are arranged linearly (Pt(1)-Pt(2)-Pt(3); bond angle is 178.3(1)°) with an average Pt-Pt bond length of 2.640 Å; this is almost identical to that of compound 5. The dppp ligands are chelating and the metal-metal bonds are not supported by any ligands. The terminal Pt atoms exhibit square-planar coordination, and the dihedral angle between the two square planes ([Pt(1)P(1)P(2)C(1)] and [Pt(3)P(3)P(4)C(2)]) is 88°. In principle, this angle could range from 0° (eclipsed) to 90° (staggered). Extended Hückel molecular orbital (EHMO) calculations of a model compound [Pt₃(HNC)₂(PH₃)₄]²⁺ indicate that the total

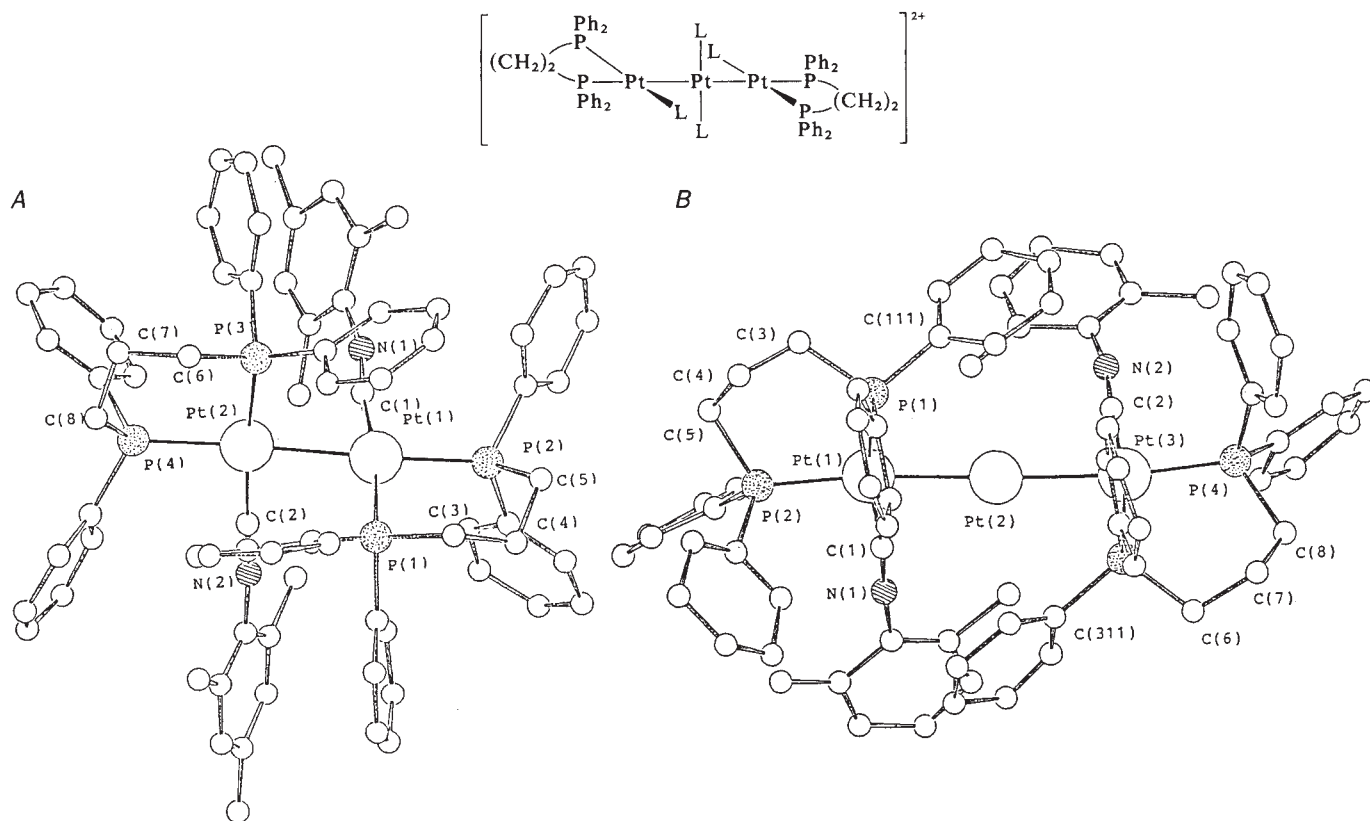


FIG. 1 A, Perspective drawing of the complex cation [Pt₂(dppp)₂(2,4,6-(CH₃)₃C₆H₂NC)₂]²⁺ (compound 2C). Crystallographic data: monoclinic, space group P2₁/a, a = 25.971(4) Å, b = 19.212(2) Å, c = 14.909(2) Å, β (a-b angle) = 101.21(1)°, Z = 4, goodness of fit R = 0.074 using 5,682 reflections

(Fo > 5σ(Fo)). B, Perspective drawing of the complex cation [Pt₃(dppp)₂(2,6-(CH₃)₂C₆H₃NC)₂]²⁺ (4B). Crystallographic data: monoclinic, space group P2₁, a = 15.491(3), b = 22.575(5) Å, c = 10.884(3) Å, β = 107.48(2)°, Z = 2, R = 0.069 using 4,373 reflections (Fo > 3σ(Fo)).

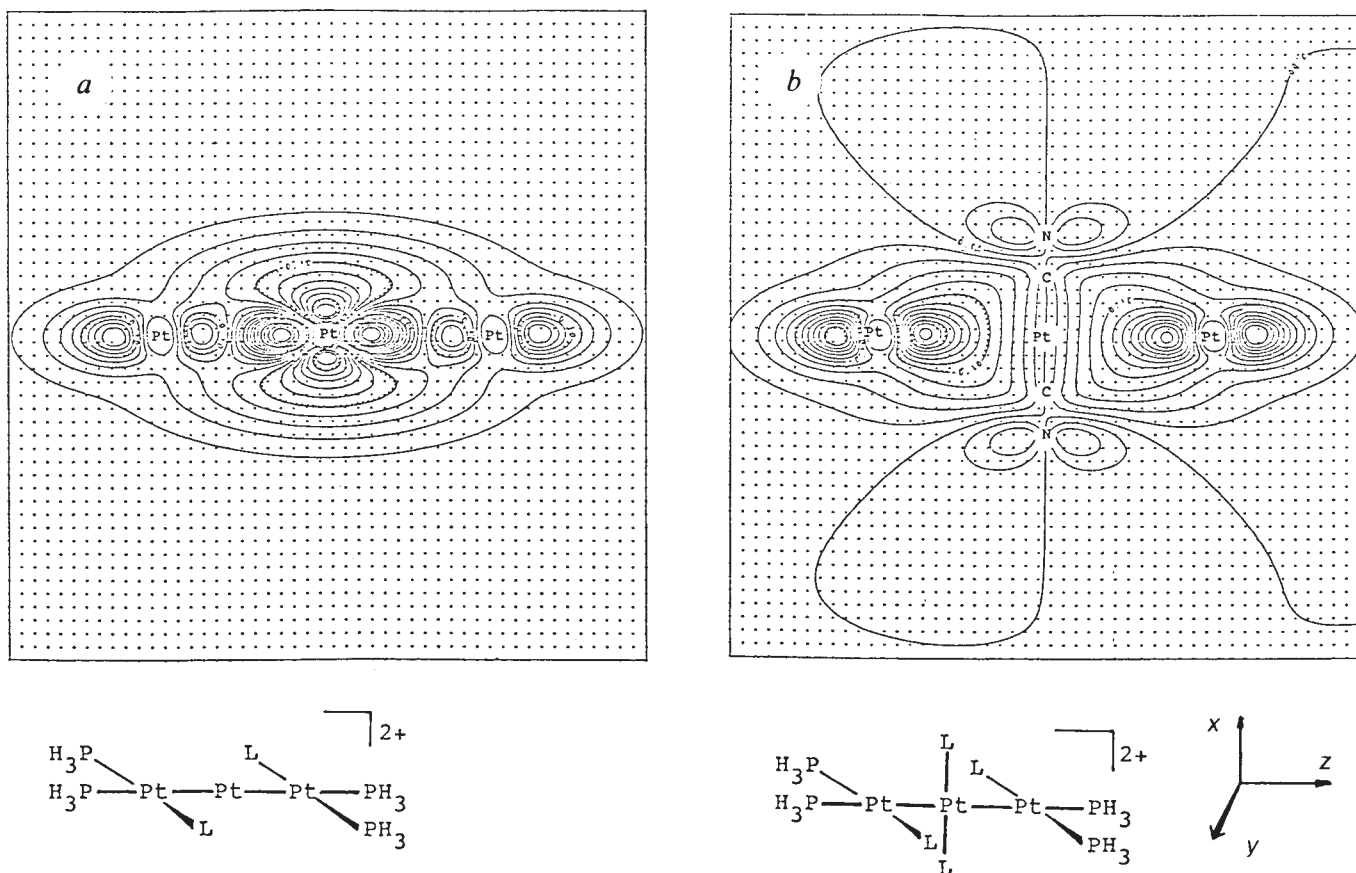


FIG. 2 Contour plots of HOMOs of compounds 6A (a) and 6B (b) (yz plane). PH_3 and HNC ligands attaching to the terminal Pt atoms are

omitted for clarity. $\text{L} = \text{HNC}$.

energy difference between these two extreme geometries is almost zero^{7,10}. That the staggered form is the one observed is therefore probably the result of steric repulsions between the two dppp ligands.

The most remarkable feature of the complex 4B is the central platinum atom, which is bound only to other Pt atoms. Otherwise, the closest neighbours of the Pt(2) atom are the dppp ligand (3.38(4) Å), and the isocyanide ligands (3.09(4) Å). These interactions are probably very weak, if they exist at all, so the Pt(2) atom is coordinatively naked. Tri-platinum complexes with 42 or 44 electrons have been observed previously^{11–13}, but the electron count of 4B is 40. To our knowledge, this is the first example of an electron-deficient tri-platinum compound.

The electronic structure of the naked platinum of 4B is of interest because of its relevance to the reactivities of the transient coordinatively unsaturated species that exist in catalytic reactions. We have performed EHMO calculations^{7,10} of the model compounds, $[\text{Pt}_3(\text{HNC})_n(\text{PH}_3)_4]^{2+}$ (6A, $n=2$; 6B, $n=4$) to elucidate the electronic structures of 4A and 4B. The energy gap between the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals of 6A is small (0.3 eV) relative to that of 6B (1.3 eV), suggesting that 4B is electronically unstable. The apparent stability of 4B is therefore probably due to steric effects of the diphosphine ligands, which overcome electronic considerations. Furthermore, there is a marked difference between the HOMOs of 6A and 6B. In 6A the HOMO consists of an interaction between the empty s orbital of the central Pt and the filled d_{z^2} orbital of the $[\text{Pt}(\text{HNC})(\text{PH}_3)_2 \cdots \text{Pt}(\text{HNC})(\text{PH}_3)_2]$ fragment (Fig. 2a), and is destabilized by a repulsive interaction with the filled d orbital of the central Pt atom. This is the dominant bonding mode, being reflected by a high charge density on the central Pt atom. The HOMO of 6B, on the other hand, consists of an interaction

between the filled d orbital (a hybrid of $d_{x^2-y^2}$ and d_{z^2}) of $[\text{Pt}(\text{HNC})(\text{PH}_3)_2 \cdots \text{Pt}(\text{HNC})(\text{PH}_3)_2]$ and the empty p_z orbital of $\text{Pt}(\text{HNC})_2$ (Fig. 2b), stabilized by the two HNC ligands. In this case, the charge of the central Pt atom is dispersed in the π^* orbitals of the HNC ligands. In other words, both bonding modes can be regarded as two σ interactions with four electrons, but in 6A, bonding electrons are localized on the central platinum atom, suggesting that the naked platinum centre of 4B is labile.

Our results show that the length of the methylene carbon chain of the diphosphine ligands dramatically influences the stereochemistry of metal-metal-bonded platinum complexes. Thus these electrochemical techniques may prove very effective in designing specific polynuclear geometries in platinum compounds. Furthermore, the complex containing a naked platinum atom serves as a model for the electronic structure of metal atoms near the surface of heterogeneous catalysts. □

Received 25 October 1989; accepted 12 February 1990.

- Muetterties, E. L. *Bull. Soc. Chim. Belg.* **85**, 451–470 (1976).
- Muetterties, E. L., Rhodin, T. N., Band, E., Brucker, C. F. & Pretzer, W. R. *Chem. Rev.* **79**, 91–137 (1979).
- Davis, S. C. & Klabunde, K. J. *Chem. Rev.* **82**, 153–208 (1982).
- Main, P., Lessinger, L., Woolfson, M. M., German, G. & Declercq, J. P. *MULTAN 78* (Universities of York and Louvain, 1978).
- Sakurai, T. & Kobayashi, K. *Rikagaku Kenkyusho Hokoku* **55**, 69–77 (1979).
- Yamamoto, Y., Takahashi, K. & Yamazaki, H. *Chem. Lett.* 201–204 (1985).
- Yamamoto, Y., Takahashi, K. & Yamazaki, H. *J. chem. Soc. Dalton Trans.* 1833–1837 (1987).
- Yoshida, T., Yamagata, T., Tulip, T. H., Ibers, J. A. & Otsuka, S. *J. Am. Chem. Soc.* **100**, 2063–2073 (1978).
- Yamamoto, T., Takahashi, K. & Yamazaki, H. *J. Am. Chem. Soc.* **108**, 2458–2459 (1986).
- Dedieu, A. & Hoffmann, R. *J. Am. Chem. Soc.* **100**, 2074–2079 (1978).
- Gubin, S. P. *Russ. Chem. Rev.* **54**, 529–555 (1985).
- Mingos, D. M. P. & Evans, D. G. *J. organometall. Chem.* **251**, C13–C16 (1983).
- Mingos, D. M. P. *Acc. Chem. Res.* **17**, 311–319 (1984).

ACKNOWLEDGEMENTS. We thank H. Yamazaki and Y. Wakatsuki of RIKEN for helpful discussions.

Relative contributions of greenhouse gas emissions to global warming

Daniel A. Lashof* & Dilip R. Ahuja†‡

* Natural Resources Defense Council, 1350 New York Avenue, Northwest Washington, DC 20005, USA

† Tata Energy Research Institute, New Delhi 110003, India

IN the past few years, many workers have noted that the combined effect on climate of increases in the concentrations of a large number of trace gases could rival or even exceed that of the increasing concentration of carbon dioxide¹⁻³. These trace gases, principally methane, nitrous oxide and chlorofluorocarbons, are present at concentrations that are two to six orders of magnitude lower than that of carbon dioxide, but are important because, per molecule, they absorb infrared radiation much more strongly than carbon dioxide. Indeed a recent study⁴ shows that trace gases are responsible for 43% of the increase in radiative forcing from 1980 to 1990 (Fig. 1). An index to compare the contribution of various 'greenhouse' gas emissions to global warming is needed to develop cost-effective strategies for limiting this warming. Estimates of relative contributions to additional greenhouse forcing during particular periods do not fully take into account differences in atmospheric residence times among the important greenhouse gases. Here we extend recent work on halocarbons^{5,6} by proposing an index of global warming potential for methane, carbon monoxide, nitrous oxide and CFCs relative to that of carbon dioxide. We find, for example, that methane has, per mole, a global warming potential 3.7 times that of carbon dioxide. On this basis, carbon dioxide emissions account for 80% of the contribution to global warming of current greenhouse gas emissions, as compared with 57% of the increase in radiative forcing for the 1980s.

In considering options for limiting global warming, the relative warming potential of given greenhouse gas emissions must be considered, rather than the relative radiative forcing of changes in atmospheric concentrations. This requires an estimate of the radiative impact of given emissions over time^{7,8}. Because greenhouse gases have different residence times, the relative cumulative impact of each gas may be quite different from its relative initial forcing. To evaluate the climate implications of current activities and proposed policies, it is desirable to have a single index for each gas⁹ that combines its radiative forcing per molecule in the atmosphere with its atmospheric residence time. Previous efforts to express emissions on a CO₂-equivalent basis have used simplified approaches that do not explicitly treat the time-dependence of CO₂ concentrations^{10,11,23}.

To provide a consistent basis for comparing emissions of different greenhouse gases, an index of global warming potential (GWP) can be defined as:

$$GWP_i = \frac{\int_0^\infty a_i(t) c_i(t) dt}{\int_0^\infty a_C(t) c_C(t) dt} \quad (1)$$

where $a_i(t)$ is the instantaneous radiative forcing due to a unit increase in the concentration of gas i , and $c_i(t)$ is the fraction of gas i remaining at time t . The corresponding values for CO₂ are in the denominator. In general, a_i is a function of the concentration of gas i and other greenhouse gases because of saturation and overlap of the respective absorption bands³. For example, the differential radiative forcing from an increase in CO₂ is about 30% greater at 350 p.p.m. than it is at 450 p.p.m. The integral can be approximated, however, by using a representative value for a_i based on a projection of concentration

changes over the residence time of gas i . Our calculations (Table 1, column 3) are based on the average radiative forcing from increasing the atmospheric concentrating CO₂ from 350 to 450 p.p.m., increasing CH₄ from 1.7 to 2.7 p.p.m., and increasing N₂O from 0.31 to 0.41 p.p.m.; the CFC radiative forcings vary in a nearly linear fashion with concentration^{3,4}.

Radiative forcing is expressed as the initial change in the Earth's radiation budget due to changes in greenhouse gas concentrations (W m⁻² p.p.m.⁻¹). Climate sensitivity is defined as the amount of global warming required to restore radiative balance and depends on uncertain climate feedbacks. Climate sensitivity is estimated to be 0.5–1.3 °C W⁻¹ m², corresponding to a warming of 2–5 °C for a doubling of CO₂ (ref. 12).

The definition of GWP given above is similar to that given by Fisher *et al.* for halocarbons⁶, which is analogous to the definition of ozone depletion potential (ODP) given by Wuebbles¹³. Both the latter definitions are based on reaching equilibrium following a step-function change in emissions, whereas our definition emphasizes the long-term implications of emissions in a given year. If residence times are constant and radiative forcing varies linearly with concentration, these definitions are identical⁶. Note that these formulations weight all future forcings equally, contrary to the case of instantaneous forcing, which neglects all future impacts. Intermediate cases in which future forcing is discounted are discussed below.

If gas i is removed from the atmosphere in proportion only to its concentration, then $c_i(t) = e^{-t/\tau_i}$, where τ_i is its average residence time. In this case, the cumulative forcing arising from this mole of gas over its atmospheric life is $a_i \tau_i$. The atmospheric retention of N₂O and the CFCs is reasonably well described by single residence times, so for these gases this approach can be used directly. Atmospheric retention of CO₂ is more complex. Rather than being destroyed, CO₂ is transferred into other reservoirs (for example, oceans and biota) from which it can return to the atmosphere. The result is that CO₂ removal cannot be described by a single residence time. Therefore, for CO₂ we follow Maier-Reimer and Hasselmann¹⁴ and let

$$c_C(t) = \sum_0^4 \alpha_j e^{-t/\tau_j} \quad ; \quad \sum_0^4 \alpha_j = 1 \quad (2)$$

The coefficients are based on Maier-Reimer and Hasselmann's fit of the response of their ocean general circulation model to an instantaneous injection of CO₂ of 25% of the current atmospheric burden (α_0 – α_4 = 0.131, 0.201, 0.321, 0.249 and 0.098; τ_0 – τ_4 = 1,000, 362.9, 73.6, 17.3 and 1.9). The original fit, however, set $\tau_0 = \infty$, which implies that $\int c_C(t) dt = \infty$. We have arbitrarily chosen τ_0 = 1,000 years, which effectively discounts the very

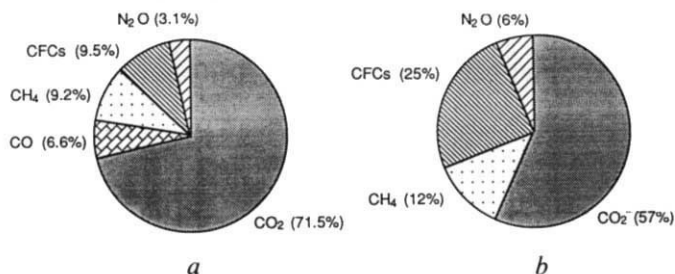


FIG. 1 *a*, Relative contributions to global warming potential in 1985 and *b*, relative contributions to greenhouse forcing added during the 1980s. The left pie is based on zero-discount rate GWPs from Table 1 and anthropogenic emissions estimates from ref. 18 as follows: 21 Pg CO₂; 800 Tg CO net carbon plus 300 Tg CO recycled carbon; 82 Tg CH₄ net carbon plus 250 Tg CH₄ recycled carbon; 5.2 Tg N₂O; 360 Gg CFC-12; 280 Gg CFC-11; 150 Gg CFC-113; 47 Gg CFC-14. Four other CFCs contributed a total of <1%. CFC residence times not given in ref. 12 were taken from refs 20 and 22, with values given as >500 years set to 500 years. The total contribution of CO₂, including net CO₂ produced from emissions originating as CO and CH₄, is ~80%. A surprisingly large contribution (1.7% of the total warming potential) was made by CFC-14 (CF₄) because of its very long residence time (set to 500 years). *b* is redrawn from ref. 4.

‡ Present address: The Bruce Company, Suite 215, 1100 6th Street, Southwest Washington, DC 20024, USA.

TABLE 1 Global warming potentials for various greenhouse gases on mole and weight bases relative to carbon dioxide

Gas	Residence time (yr)	(Molar basis)			(Weight basis)	
		Instantaneous forcing (W m^{-2} p.p.m. $^{-1}$)	Cumulative forcing (W m^{-2} yr Pmol^{-1})	Global warming potential	Cumulative forcing (W m^{-2} yr Pg^{-1})	Global warming potential
CO_2	230	0.015	19	1.0	0.42	1.0
CO	(2.1)	(0.65)	26	1.4	0.94	2.2
CH_4	(14.4)	(0.65)	71	3.7	4.4	10
N_2O	160	3.8	3,400	180	77	180
HCFC-22	15	190	15,000	810	180	410
CFC-11	60	220	74,000	4,000	540	1,300
CFC-12	120	280	190,000	10,000	1,600	3,700

Residence times are derived as follows: CO_2 (ref. 14), CH_4 and N_2O (ref. 20) and CFCs (ref. 6). The residence times of CO_2 and CH_4 are adjusted as discussed in the text. CO is ascribed a residence time equivalent to 0.22 times that of methane (see text). The cumulative forcing and GWPs for CO and CH_4 are based on net carbon and therefore include the warming potential of CO_2 . Instantaneous radiative forcings are based on the parameterized model given in ref. 3, except for HCFC-22 which is calculated from tables in ref. 4. Values that depend on concentration are based on increasing CO_2 from 350 to 450 p.p.m., increasing CH_4 from 1.7 to 2.7 p.p.m. with a 70% amplification (see text), and increasing N_2O from 0.31 to 0.41 p.p.m. Climate sensitivity is estimated to be $0.5\text{--}1.3^\circ\text{C W}^{-1}\text{ m}^2$, corresponding to a warming of $2\text{--}5^\circ\text{C}$ for a doubling of CO_2 (ref. 12).

long-term retention of a small fraction of the original emissions. Results are quite sensitive to this choice for τ_0 . The integral of equation (2) is $\sum \alpha_j \tau_j$, which yields the effective residence time of 230 years for CO_2 given in Table 1. Adopting an effective residence time of 500 years as suggested by Edmonds and Wuebbles¹⁵ (equivalent to letting $\tau_0 = 3,000$ years) would reduce the GWPs in Table 1 by a factor of two. Shorter effective residence times would be obtained if future warming is discounted (see below).

The GWP of methane is influenced by chemical interactions in the atmosphere that should be taken into account. First, we note that all carbon emitted into the atmosphere as methane is eventually oxidized to CO_2 and H_2O . For methane emitted from the decomposition or burning of renewably produced organic matter, the CO_2 generated recycles carbon that was removed from the atmosphere during photosynthesis (recycled carbon). The entries in Table 1, however, are based on net carbon emissions (from fossil fuels or from net deforestation), for which the cumulative forcing from one mole of CO_2 adds to the cumulative forcing from one mole of methane *per se*. In addition, methane emissions tend to increase tropospheric ozone and stratospheric water vapour concentrations, perhaps enhancing the direct radiative forcing from methane by $\sim 70\%$ (ref. 16). Finally, to account roughly for increases in the residence time of methane resulting from $\text{CH}_4\text{--CO--OH}$ coupling, we assume that each mole of methane emitted increases the residence time of one mole of methane by 50% (refs 16, 17).

The GWP of CO is closely related to that of methane. Carbon monoxide itself is not a greenhouse gas, but it too is oxidized to CO_2 in the atmosphere, implying that for net carbon its GWP must be at least 1. Additional forcing is contributed through the coupling of $\text{CH}_4\text{--CO--OH}$ as CO emissions increase the residence time of methane and therefore its atmospheric burden. Model calculations¹⁷ suggest that each mole of CO emissions increases the residence time of one mole of CH_4 by approximately 20%. Each mole of 'net-carbon' CO emissions is therefore ascribed the cumulative forcing of 0.2 times that of one mole of CH_4 plus the cumulative forcing which arises from one mole of CO_2 .

Estimates of cumulative radiative forcing caused (or averted) by each petamole (10^{15} mole) of gas emitted (or removed) in order of increasing global warming potential are shown in column 4 of Table 1. Column 5 shows the GWPs; for CO_2 the GWP is 1 by definition. The warming potential of carbon monoxide is 40% higher than that of CO_2 and, despite the adjustments which increase the forcing from methane emissions, we find that the GWP of methane is only 3.7, much lower than the ratio of instantaneous radiative forcings shown in column 3 (which have been adjusted as described above). Nitrous oxide is 180 times as effective as CO_2 , and HCFC-22 oxide is 180

times as effective as CO_2 , in causing cumulative global warming. HCFC-22, which is a leading potential substitute for other CFCs because of its relatively low capacity to destroy ozone, has a GWP of 810, much higher than the naturally occurring greenhouse gases, but lower than CFC-11 ($\sim 4,000$) and CFC-12 ($\sim 10,000$)—the two most commonly used CFCs that it might replace. Our results for the halocarbons are consistent with the values in Fisher *et al.*² to within 20%, when common reference residence times are used.

Results based on emissions of one petagram (Pg) of each gas are shown in column 6 of Table 1, with the corresponding weight-based index in column 7. This index will be higher than the GWP for those gases that have a lower molecular weight than CO_2 and vice versa. Although the GWP is a physically

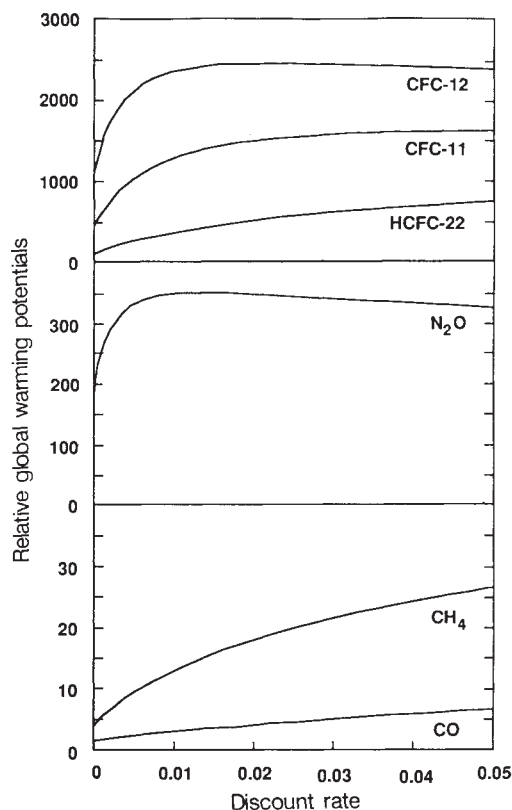


FIG. 2 Global warming potentials relative to CO_2 as a function of discount rate. The effective residence time of CO_2 decreases disproportionately with increasing discount rate because it is expressed as the sum over multiple exponentials. The GWPs of CO and CH_4 are based on net carbon emissions.

more meaningful index (accurately reflecting the implications of burning rather than venting one mole of methane, for example) we have presented both results because emissions data are frequently reported on a weight basis. (For consistency we have used the full molecular weight of CO_2 in columns 6 and 7, rather than the carbon weight which is commonly used in the carbon-cycle literature. For the carbon cycle gases the molar GWPs are identical to GWPs on a carbon weight basis.)

Current radiative forcing may be considered more important from a policy viewpoint than radiative forcing occurring in the distant future because, for example, of the possibility that new technologies will emerge to solve the problem. Intergenerational equity issues and the likelihood that the value placed on limiting climate change will grow with economic output, however, argues against discounting future forcing. Discounting can be accommodated by multiplying future forcing by e^{-rt} , where r is called the discount rate and is analogous to an interest rate. For a gas with a single decay time, τ , its effective residence time reduces to $\tau/(1+r\tau)$. For a gas with multiple decay rates like CO_2 , discounting reduces the equivalent residence time disproportionately, from $\sum \alpha_j \tau_j$ to $\sum \alpha_j \tau_j / (1+r\tau_j)$. Applying a discount rate of 1% per year, for example, reduces the effective residence time of CO_2 from 230 to 45 years. As higher discount rates are assumed, the significance of residence time diminishes and the GWP approaches the respective relative scaled radiative forcing per molecule for each gas (Fig. 2). A similar result can be obtained by integrating equation (1) over a finite time interval, but this implies a discontinuity in weighting future forcing.

Neglecting for the time being the contributions from NO_x and ozone, this framework can be used to calculate the contributions to global warming potential by different gases, regions and activities. Using data compiled by the US Environmental Protection Agency¹⁸, we have estimated the relative contributions of the various greenhouse gases for the year 1985 using the zero discount rate GWPs from Table 1. These differ substantially from the contributions calculated on the basis of concentration increases during the 1980s (Fig. 1)³. The contribution of CO_2 dominates, in spite of its low radiative forcing, because of the magnitude of its emissions and atmospheric residence time. Indeed, the total contribution of CO_2 , including net CO_2 produced from emissions originating as CO and CH_4 , is ~80% of the warming potential generated in 1985.

The largest source of uncertainty in our results stems from the assumed effective residence time of CO_2 . The results are sensitive not only to the long-term behaviour of a small fraction of the initial emissions, but also to the underlying assumptions in the carbon-cycle model and the scenario of future CO_2 emissions¹⁴. There is also considerable variation in the estimated residence times of some of the other gases (for example, CH_4 ¹⁹, HCFC-22^{6,20}) and in how feedbacks may change these in the future. Uncertainty in the radiative forcings per unit change in concentration arises primarily from accounting for the warming amplification from ozone and stratospheric water vapour associated with methane oxidation, and from choosing representative future concentrations¹⁶. The impact of CH_4 -CO-OH coupling on the residence time of methane is also nonlinear and depends on emissions of NO_x and other compounds²¹. The values presented here are intended to establish an order-of-magnitude comparison and stimulate further work to refine the GWP estimates. Analyses of different scenarios will continue to be necessary as a complement to the use of GWP point estimates.

The formulation developed here will help to address policy questions regarding the relative amounts of rational expenditures on different mitigating strategies. Suppose that one can estimate the cost of reducing, say, one Pmol of emissions of a greenhouse gas. Using information in column 4 of Table 1, the costs of averting a unit of cumulative forcing by that option can be calculated. As the potential for reduction in greenhouse gas emissions from any option is limited, a 'supply curve' is needed to rank in order the costs of different options along with the

quantity of radiative forcing each option can prevent. Then, for any given amount of expenditure the most cost-effective options can be undertaken first. Alternatively, if it were decided that an expenditure of \$1 is justifiable to reduce (remove or prevent), say, 1 kmol of CO_2 emissions in order to mitigate global warming, then it would be rational to spend $\$1 \times \text{GWP}_i$ to reduce emissions of gas i by 1 kmol. Higher discount rates will have the effect of justifying higher expenditures to reduce emissions of other greenhouse gases relative to those of CO_2 . Estimates of the relative costs of different options must be developed with great caution, however, because the most attractive options will often be those that have significant other benefits besides reducing greenhouse gas emissions (for example, increasing energy efficiency).

The index of global warming potential developed here should prove useful in clarifying the relative contributions to global warming of different countries and different activities and should make it easier to develop cost-effective emissions policies at both national and international levels. A prerequisite for using this index as a basis for legal requirements, however, is the availability of reliable methods to establish current emissions and to monitor changes. □

Received 24 August 1989; accepted 26 February 1990.

1. Laci, A., Hansen, J., Lee, P., Mitchell, T. & Lebedeff, S. *Geophys. Res. Lett.* **8**, 1035-1038 (1981).
2. Ramanathan, V., Cicerone, R. J., Singh, H. B. & Kiehl, J. T. *J. geophys. Res.* **90**, 5547-5566 (1985).
3. Hansen, J. et al. *J. geophys. Res.* **93**, 9341-9364 (1988).
4. Hansen, J., Laci, A. & Prather, M. *J. geophys. Res.* **94**, 16417-16421 (1989).
5. Rogers, J. & Stephens, D. *J. geophys. Res.* **90**, 2423-2428 (1988).
6. Fisher, D. et al. *Nature* **344**, 513-516 (1990).
7. Swart, R., de Boois, H. & Vellinga, P. in *The Full Range of Responses to Anticipated Climatic Change* Ch. 9, 137-159 (United Nations Environment Program and The Beijer Institute, Stockholm, 1989).
8. Smith, K. & Ahuja, D. *Clim. Change* (in the press).
9. *Noordwijk Declaration on Atmospheric Pollution and Climatic Change* (Netherlands Ministry of Environment, The Hague, November 1989).
10. Deluchi, M., Sperling, D. & Johnston, R. *Transportation Fuels and the Greenhouse Effect* (University of California, University Energy Research Group, UER-182, 1987).
11. Okken, P. & Kram, T. *CH₄/CO-emission from fossil fuels global warming potential ESC-WR-89-12* (ECN, Petten, The Netherlands, June 1989).
12. Dickinson, R. & Cicerone, R. *Nature* **319**, 109-114 (1986).
13. Wuebbles, D. *The Relative Efficiency of a Number of Halocarbons for Destroying Stratospheric Ozone* Report UCID-18924 (Lawrence Livermore National Laboratory, Livermore, 1981).
14. Maier-Reimer, E. & Hasselmann, K. *Clim. Dyn.* **2**, 63-90 (1987).
15. Edmonds, J. & Wuebbles, D. *A Primer on Greenhouse Gases* (US Department of Energy, NBB-0083, Washington, DC, 1988).
16. Lashof, D. *Clim. Change* **14**, 213-242 (1989).
17. Thompson, A. & Cicerone, R. *J. geophys. Res.* **91**, 10853-10864 (1986).
18. Lashof, D. & Tirpak, D. *Policy Options for Stabilizing Global Climate* Draft Report to Congress (US Environmental Protection Agency, Washington, DC, 1989).
19. Cicerone, R. & Oremland, R. *Global Biogeochemical Cycles* **2**, 299-328 (1988).
20. Prather, M. *An Assessment Model for Atmospheric Composition* NASA Conference Publication 3023 (NASA, Washington, DC, 1989).
21. Thompson, A. et al. *Atmos. Envir.* **23**, 519-532 (1989).
22. Ramanathan, V. et al. *Rev. Geophys.* **25**, 1441-1482 (1987).
23. Okken, P. *Energy Policy* (in the press).

ACKNOWLEDGEMENTS. The work of D.R.A. on this project was supported by the Office of Policy Analysis, US Environmental Protection Agency. We thank K. Smith and D. Tirpak for stimulating and encouraging this work. We are grateful to T. Wigley for a very thorough and constructive review of the manuscript. Helpful comments were also provided by M. Deluchi, M. Gibbs, M. Prather, P. Okken and R. Swart.

Microatolls and recent sea level change on coral atolls

Colin Woodroffe* & Roger McLean†

*Department of Geography, University of Wollongong, New South Wales 2500, Australia

†Department of Geography and Oceanography, Australian Defence Force Academy, Canberra, Australian Capital Territory, 2600, Australia

MICROATOLLS are colonies of corals, commonly *Porites*, which are dead on top but living around their perimeter, and are found in intertidal environments on coral atolls. They can grow to several metres in diameter. Their upward growth is constrained by sea level through prolonged exposure at the lowest spring tides^{1,2}, and

their dead upper surfaces have been limited by past sea levels. They act as natural recorders of sea level, which is of particular significance for coral atolls thought to be susceptible to inundation and erosion if sea level rises in response to global warming. X-radiographs of vertical slices through microatolls from the Maldives and Cocos (Keeling) Islands (Indian Ocean) and Kiribati (Pacific Ocean) record sea-level fluctuations over the past few decades. There is a high degree of reproducibility between adjacent corals, although on Cocos we noted geographical variation in the pattern of change around the atoll. The majority of microatolls sampled on these atolls record a slight fall in sea level over the past ten years.

The response of microatolls to changes in sea level is shown schematically in Fig. 1. Where massive corals are subtidal, they usually grow radially, adopting a hemispherical form (Fig. 1a). The upper limit to coral growth occurs at a level (λ) where prolonged exposure at the air/water interface during low spring tides prevents further upward growth¹. When a coral reaches that level (λ), upward growth ceases but horizontal growth continues, and the coral adopts a microatoll form (Fig. 1b). If a microatoll then experiences a rising sea level, it will resume upward growth in an effort to 'keep-up'; it will tend to overgrow its dead surface as shown in Fig. 1c. Alternatively, if sea level falls the coral will continue its lateral extension at lower levels, and if it falls episodically one or more terraces may develop (Fig. 1d). Where there are fluctuations in sea level, periods of higher sea level will be marked by upward growth extending inward over the dead surface; subsequent periods of lower sea level will lead to cessation of this upward growth but continued horizontal extension at some lower level (Fig. 1e). These responses are generalized, but we have observed each of the microatoll forms shown in Fig. 1 in a variety of reef settings.

Variations in coral skeletal density can be detected on X-radiographs of coral slices, and annual growth rates and past growth forms can be interpreted from the banding³⁻⁵. For *Porites* annual growth rates in the range 5–25 mm yr⁻¹ have been demonstrated^{6,7}. The microatoll growth form has been recognized in fossil corals, and radiometric dating of these corals has been used to reconstruct Holocene sea-level change⁸⁻¹⁰. Surface form of microatolls has been used to detect and date seismic events on tectonically active coasts¹¹. On the Great Barrier Reef, microatolls are often moated in pools behind storm ridges and their upper surfaces record the history of moated water levels^{12,13}. We demonstrate that, in some circumstances, especially on tectonically inactive mid-plate coral atolls where the tidal range is small (and thus interannual variations in sea level are small relative to coral growth rate) and which rarely experience catastrophic storms (thus moating is rare), dead upper surfaces of live microatolls can be used to reconstruct sea-level changes over recent years.

Figure 2 shows an X-radiograph of a vertical slice through a *Porites* microatoll from the seaward reef flat of Veymandoo island on Kolamadulu atoll, Maldives (maximum tidal range

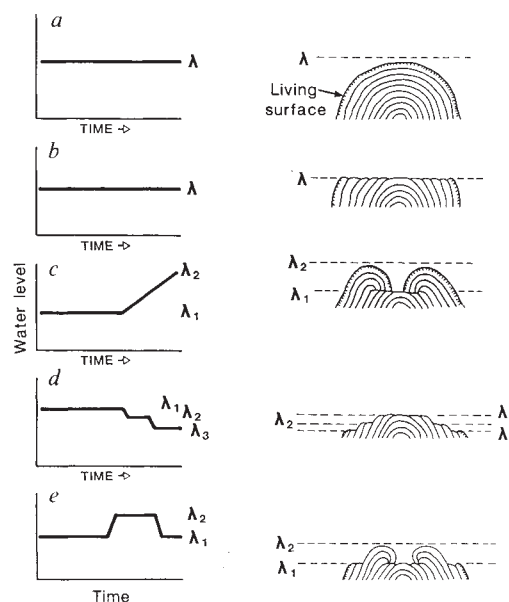


FIG. 1 The response of microatolls to different patterns of sea-level change: a, sea level not limiting, coral hemispherical; b, constant water level; c, water level constant then rising; d, water level falling episodically; e, fluctuating water level.

1.4 m). The pattern of density banding is symmetrical except where damaged through bioerosion or fracture of the top in sectioning, and indicates that the coral grew initially as a hemisphere and then, after reaching the upper limit for coral growth, extended laterally. For several years, it did not grow above a level below the present limit to coral growth (c in Fig. 2). There are two periods, marked by concentric rings on the upper surface of the coral, during which the coral grew upward and began to overgrow its dead surface, under a temporarily higher limit to coral growth (d, e in Fig. 2). Both upward phases of growth were arrested by a subsequent lowering of the limit (λ). Several fluctuations in sea level are indicated (compare Fig. 1e), but it seems that there has been little if any net change in sea level over the several decades for which this microatoll has been growing.

Large *Porites* microatolls, up to 2.5 m diameter, are common on the broad seaward reef flats of the atolls of western Kiribati, central Pacific. All 34 of the radially symmetrical microatolls surveyed at Kuria and Abemama in May 1989 showed a similar concentric pattern of surface morphology and inferred sea-level history over the 50–100-year lifetime of the corals. Typically, the living perimeter comprises a narrow 2–4-cm step which rises an equivalent height inward to a distinct asymmetrical rounded ridge (Fig. 3). The remainder of the dead microatoll surface extends a further 50–120 cm to the centre, and is essentially planar and at a level 1–2 cm above the present living edge.

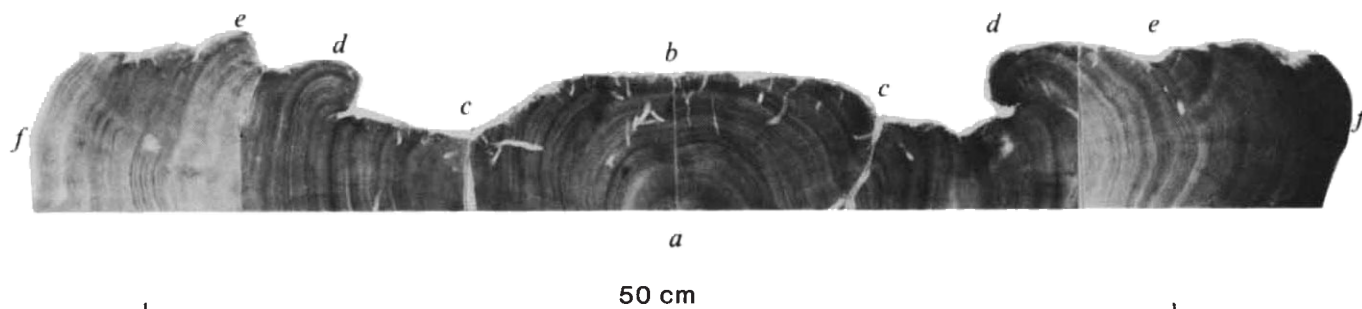


FIG. 2 X-radiograph of a vertical slice of a *Porites* microatoll from Veymandoo, Kolamadulu, Maldives. Initially the coral had a hemispherical growth form (a) until it reached the limit (λ) to growth (b). It grew out laterally for

a period limited by a lower water level (c). Two phases of upward growth can be identified (d and e). The living surface of the coral is represented by f. (The left-hand side of the coral has fractured at point e.)

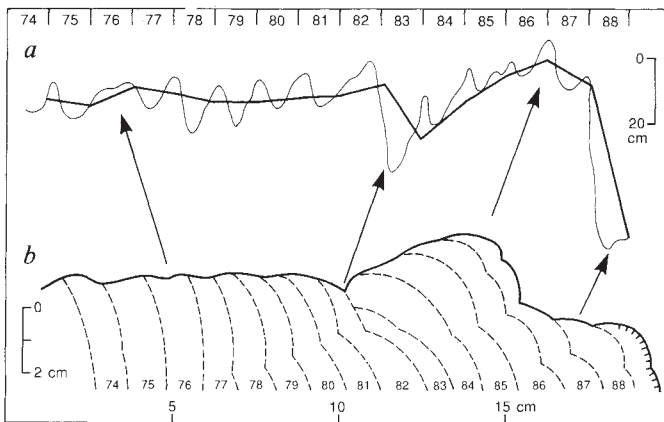


FIG. 3 Comparison of *a*, yearly mean (bold) and 3-month running mean (fine) of monthly mean sea level at Tarawa, 1974–88 (K. Wyrski, unpublished data); and *b*, pattern of annual coral growth based on X-radiography of *Porites* microatoll slice collected from Abemama, Kiribati. Living edge on right.

Figure 3 compares tide gauge records from Tarawa (mean spring tidal range 1.65 m) with sea-level changes deduced from surface morphology and the temporal pattern of coral growth (based on X-ray density banding) of the outer 20 cm of a representative microatoll from Abemama. Increments of coral growth seem to be uniform without change in the surface level of the coral for the period 1974–82. In 1982–83, a hiatus in growth occurred, presumably associated with sea-level variations during the 1982–83 El Niño event, which resulted in a lowering of the living edge in 1983. The subsequent trend of rising sea level resulted in upward growth which stopped abruptly in 1987, after which coral growth recommenced at a lower level leaving a distinct step between the 1987 and 1988–89 growth levels (Fig. 3). Negative sea-level anomalies have been reported from the Tarawa tide station throughout 1988–89. Although a preliminary analysis indicates a crude correlation between the temporal pattern of mean sea level from the gauge and recent microatoll growth, microatolls produce substantially more subdued sea-level records in which short-lived or low-magnitude variations in sea level are not represented. Moreover, microatolls respond not to mean sea level itself, but to interannual variations in the limit to coral growth (λ).

On the Cocos (Keeling) Islands in the eastern Indian Ocean (maximum tidal range 1.4 m) *Porites* microatolls are found ~0.3–0.4 m above chart datum (lowest astronomical tide) in three environments (Fig. 4a): (1) open reef flat, (2) inter-island channels, and (3) the lagoon. Many microatolls were examined on Cocos and more than 30 were sampled; the majority show evidence of a recent fall in sea level. Within fields of microatolls from inter-island channels on the eastern margin of the atoll, adjacent specimens show similarity in form with terraces discernible on their upper surface, and banding indicates that they have developed as a result of synchronous water-level fall (see 61, 62 and 63, Fig. 4b). A similar pattern of change is recorded on a nearby lagoonal microatoll (161, Fig. 4b). A less pronounced emergence is seen on the reef flat at the southern end of the atoll (810 and 811, Fig. 4b). Marked differences in hydrodynamic conditions within and between the three environments exert an influence on microatoll form and presumably account for some geographical variation in apparent sea-level change around the atoll. Under-surface banding patterns reveal a complex history of partial sediment burial and re-excitation of some microatolls, but those sampled were on a firm substrate and had not collapsed or been tilted (as occurs on more storm-affected reefs¹).

Caution needs to be observed in interpreting past sea-level change from the upper surface of microatolls. First, a microatoll form may in some cases be a response to other factors (sediment accumulation, bleaching of upper surfaces or nutrient shortages) and not solely a response to water level. Water level is implicated where exposure of microatolls occurs at lowest spring tides (Maldives, Kiribati), or where levelling has shown that the upper surface occurs at the same height in several separate microatoll fields (Cocos). Second, the effective water level which limits microatoll growth is difficult to define and may vary within atolls and regionally with the time of day at which extremely low tidal levels occur (related to phase of the solar semi-diurnal tide). It is presumably dependent not just on the still-water position of the tide, but also on local hydrodynamic factors such as magnitude and persistence of waves and current action (likely to be minimal in relatively storm-free situations such as the Maldives and Kiribati, of more importance in higher-energy trade-wind settings like Cocos, and a serious complication in stormy settings where moating and microatoll tilting may be common). Third, density banding is not always clear in corals, particularly in

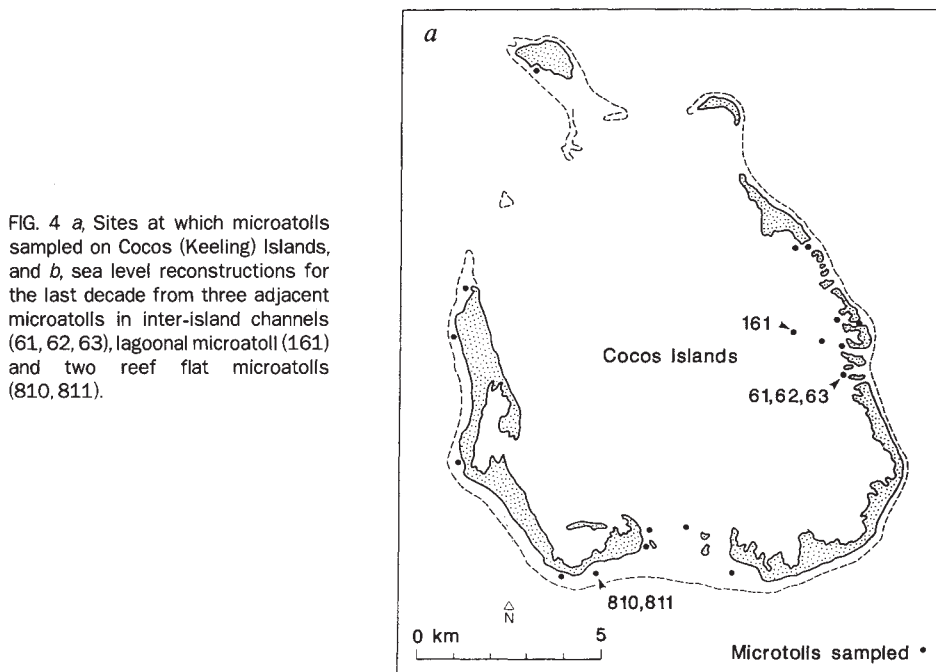
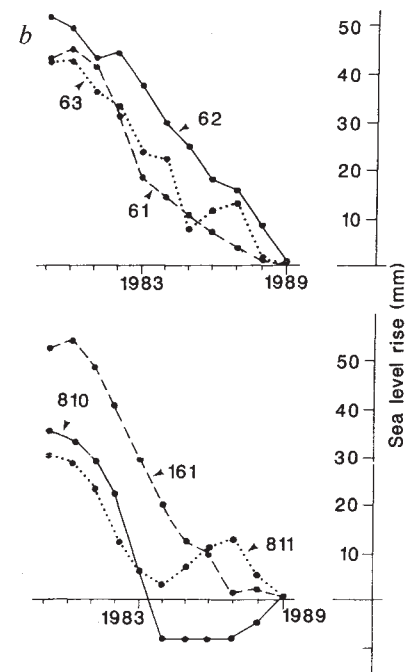


FIG. 4 *a*, Sites at which microatolls sampled on Cocos (Keeling) Islands, and *b*, sea level reconstructions for the last decade from three adjacent microatolls in inter-island channels (61, 62, 63), lagoonal microatoll (161) and two reef flat microatolls (810, 811).



intertidal corals, nor are bands unambiguously annual. Banding was poorly defined in some Cocos microatolls, but many specimens showed evidence of severe stress associated with an atoll-wide fish and coral kill documented in 1983, and this stress band served as an important marker in determining microatoll chronology. Fourth, dead upper surfaces of microatolls undergo bioerosion and the interior of large microatolls may be highly degraded and represent only a minimum past sea level. Finally, the rate of response of microatolls to sea-level rise is limited by coral growth rate, which is likely to mean that microatolls record interannual variations in sea level more accurately where tidal range is small rather than large.

Large microatolls on the reef flats of coral atolls indicate that there has not been any substantial net rise in sea level over the several decades taken to grow to such size. Indeed, many of those we sampled suggest a slight net fall in sea level over the past decade, most pronounced in Kiribati and Cocos where microatoll growth is presently limited to lower levels than previously. Geographical variations in sea-level change around an atoll may be determined where suitable microatolls of sufficient diameter are found. Microatoll records offer both important geographical supplements to tide gauges and the immediate opportunity of reconstructing past sea levels in areas where tide gauges are not installed. They also offer a natural way to monitor future sea-level change on these remote islands. □

Received 9 November 1989; accepted 9 February 1990.

1. Scoffin, T. P. & Stoddart, D. R. *Phil. Trans. R. Soc. Lond. B* **284**, 99–122 (1978).
2. Stoddart, D. R. & Scoffin, T. P. *Atoll Res. Bull.* **224**, 1–17 (1979).
3. Knutson, D. W., Buddemeier, R. W. & Smith, S. V. *Science* **177**, 270–272 (1972).
4. Dodge, R. E. & Vaisnys, J. R. *Nature* **258**, 706–708 (1975).
5. Hudson, J. H., Shinn, E. A., Halley, R. B. & Lidz, B. *Geology* **4**, 361–364 (1976).
6. Isdale, P. *Nature* **310**, 578–579 (1984).
7. Scoffin, T. P., Tudhope, A. W. & Brown, B. E. *Coral Reefs* **7**, 169–178 (1989).
8. McLean, R. F., Stoddart, D. R., Hopley, D. & Polach, H. *Phil. Trans. R. Soc. Lond. A* **291**, 167–186 (1978).
9. Chappell, J. *Nature* **302**, 406–408 (1982).
10. Woodroffe, C. D., McLean, R. F., Polach, H. & Wallensky, E. *Geology* **18**, 62–66 (1990).
11. Taylor, F. W., Frohlich, C., Leocle, J. & Strecker, M. *J. geophys. Res.* **92**, 4905–4933 (1987).
12. Hopley, D. & Isdale, P. *Search* **8**, 79–81 (1977).
13. Hopley, D. *Geomorphology of the Great Barrier Reef* (Wiley, New York, 1982).

ACKNOWLEDGEMENTS. We thank the National Geographic Society and the Australian Research Council for funding. We thank Cocos Islands Administration, Cocos Islands Council, Government of Republic of Maldives and the Commonwealth Secretariat for their support.

Vesuvius/Avellino, one possible source of seventeenth century BC climatic disturbances

J. S. Vogel*[‡], W. Cornell[†], D. E. Nelson*
& J. R. Southon*[‡]

* Department of Archaeology, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

[†] Geological Survey of Canada, 601 Booth Street, Ottawa, Ontario K1A 0E8, Canada

THE eruption of Vesuvius in AD 79 (as described by Pliny) is the archetype^{1–4} of explosive ‘plinian’ eruptions which can cause recurrent local destruction and which may affect global climate through aerosol emissions^{5–7}. Studies of the past eruptions of such volcanoes require accurate age determinations, especially if the eruptions are to be correlated with distant events. Here we use materials from two plinian eruptions of Vesuvius, the AD 79 and the preceding Avellino^{1,8} event, to test the ¹⁴C methods that often provide these ages. Our ¹⁴C ages for charred samples and corrected soil reser-

voirs buried by the Avellino eruption averaged to 3,360 ± 40 BP (1617–1703 cal BC), 200–500 years later than the original ¹⁴C ages of soil humic carbon^{9,10} and total organic carbon¹¹. Furthermore, a re-evaluation of the ¹⁴C data for the Mount St Helens Y_n and Aniakchak II eruptions suggests that these major plinian eruptions may also have occurred in the seventeenth century BC. The ¹⁴C ages for these three eruptions are identical, within uncertainties, to that of the ‘Minoan’ eruption of Thera (Santorini), which has often been correlated with mid-seventeenth century BC disturbances recorded in tree-ring series^{12,13} and ice-core layers¹⁴. Additional information is still required to distinguish which, if any, of the eruptions relate to these records.

Radiocarbon ages for eruptions are frequently taken from palaeosols and peats because other carbonaceous materials are not often found in or below the ash falls in sufficient quantity for decay counting. Such ¹⁴C ages can be useful if thick layers of adsorptive pumice and ash protect the soils and peats from the rejuvenation that occurs in genetic soils¹⁵. These materials, however, represent reservoirs integrated over uncertain times, and corrections must be applied to obtain the date of burial. Preferred dating materials are short-growth macrofossils and plant detritus which have been buried by the eruption. However, charcoal, which is resistant to decay, must be chosen carefully from strata in which redeposition of material, either young or old, is unlikely.

At least six plinian eruptions of Vesuvius¹⁶ occurred in the 15,000 years before the eruption¹⁷ that buried Roman cities in AD 79. The youngest of these previous eruptions was the Avellino, for which ¹⁴C ages ranging from 3,510 to 3,870 yr BP have been obtained^{9–11}. Our primary Avellino eruption samples were collected from a quarry near Ottaviano on the northeast flank of Vesuvius (40° 51' N, 14° 29' E) where five plinian pumice falls (including that from the AD 79 eruption) are preserved. Because the original dates were from palaeosols, we selected 4-cm³ aliquots from A and B_w palaeosol horizons below the Avellino pumice layer, which is 130 cm thick and is topped by ashfalls and pyroclastic surges and flows. A small-diameter piece of charcoal (twig?) in this overlying ash was probably from a tree branch which protruded through the thick pumice and was eroded by a surge. The AD 79 deposit is thinner (20 cm), but distinct, at this quarry and capped a sampled B horizon sol. Although quarrying has removed overburden to within 30 cm of the AD 79 pumice, contaminating organic compounds are unlikely to have penetrated the adsorptive, lapilli-bearing ash in the short time (~5 yr) between quarrying and collection¹⁸. Two other samples came from a quarry near Pozzelle on the east-southeast slopes of the volcano where the AD 79 and possibly Avellino-equivalent deposits occur. The Avellino deposit seems to be represented only by pyroclastic surges or flows. A small sample from this stratum disintegrated into stringy pieces during preparation, suggesting that it was a charred plant stem. Charcoal was also selected from a B horizon sol 30 cm below the plant stem. Although all samples were originally collected for other geochemical studies, dating was possible because of the small-sample capabilities of accelerator mass spectrometry (AMS). The samples were prepared and dated using our normal protocols¹⁹.

The ages of the Avellino materials are shown in Table 1. The twig (RIDD1343) and the plant stem (RIDD1338) gave ages that are significantly younger than the original dates assigned to the eruption. Their stratigraphic positions in later eruption surges after the pumice fall preclude any redeposition of old charcoal, and their sizes represent short growth periods. The charcoal sample (RIDD1342) that agreed with previous Avellino dates was well below the Avellino correlative units at Pozzelle. Redeposition from overlying units was unlikely, as an undisturbed water-laden unit which had supported a dense snail population lay between the Avellino strata and this sample.

We used wood and charcoal from the AD 79 event to test our accuracy in dating these samples (Table 2). A small, unidentified

[‡] Present address: Center for Accelerator Mass Spectrometry, L-397, Lawrence Livermore National Laboratory, Livermore, California 94550, USA.

TABLE 1 Avellino eruption materials and radiocarbon dates

Identification	Lab. no. RIDDL-	$\delta^{13}\text{C}^*$ (‰)	Age† (yr BP)	Error‡ (±)	Corrected ages§ yr BP	Error
Pozzelle quarry						
Plant stem in surge ash	1338	-26.1	3,300	80	3,300	80
Charcoal in B horizon 30 cm below	1342	(-25)	3,760	80		
Ottaviano quarry						
Twig charcoal above pumice	1343	-23.8	3,430	50	3,430	50
Palaeosol A horizon, 0-17 cm						
charcoal (56 µg)	1350	(-25)	4,040	260		
hydrolysate	1351	-23.5	3,330	55	3,130	210
humics	1352	-25.5	3,715	50	3,270	160
humins	1353	(-25)	3,950	110		
Palaeosol B horizon, 17-31 cm						
charcoal (125 µg)	1354	(-25)	4,180	150		
hydrolysate	1355	-23.5	3,240	70	3,040	210
humics	1356	-25.3	3,790	70	3,340	160
humins	1357	-25.4	3,905	90		

* Isotope fractionation corrections were made from $\delta^{13}\text{C}$ measurements on the larger samples. Assumed values for other samples are in parentheses. $\delta^{13}\text{C}$ measurements are referenced to PDB standard in units per mil (‰).

† All dates are reported using the international conventions of Stuiver and Polach³⁹.

‡ Uncertainties are standard deviations of multiple (≥ 3) measurements expressed as 1σ .

§ No correction applied to small charcoals. Reservoir ages of 450 ± 150 ^{14}C yr subtracted from humic ages, and 200 ± 200 ^{14}C yr subtracted from hydrolysate ages. Humins ages were not used.

charcoal piece (RIDDL-1346) from soil at the Pompei necropolis agreed with the known date of the AD 79 eruption. We dated charred and uncharred portions of a lintel beam removed from a surge level at Herculaeneum to test for any effect due to contact with hot pyroclastic material. The data were concordant at 60-130 calibrated years before the eruption, consistent with the size and use of the beam. A second large beam in Herculaeneum surge material was dated to 145 ± 60 years before AD 79. Architectural beams are not ideal calibration samples, but these reasonable ages indicate an accuracy of better than 100 years, with no systematic bias toward young dates. The multiple measurements on the lintel were reproducible within our stated precision.

The Avellino palaeosols were chemically fractionated for dating. Studies of genetic soils find different reservoir ages for separate chemical fractions²⁰⁻²²; a 6M HCl hydrolysate often has the shortest mean residence time; the commonly dated humic acids are many hundred years old; and the refractive humin can contain very old carbon. Palaeosols under thick volcanic strata simulate isolated surface soils, so we used similar fractions in this study (Table 1). Humin contained the expected old carbon, whose age at burial we deemed too uncertain to use. Charcoal flecks were old and probably redeposited. Humin and charcoal may have contributed to a previous age of $3,760 \pm 70$ (Gif-4517)¹¹ on total palaeosol organic carbon. The ^{14}C concentrations in our humic fractions (RIDDL-1352, 1356) agreed with the previous value for humics, $3,870 \pm 50$ BP (R-938)¹⁰. The measured ages of the hydrolysate fractions agreed with our charcoal dates.

To understand the Avellino palaeosols, we measured the ^{14}C reservoir ages of local soils which were buried by the AD 79 eruption. As variations exist between soil types, we measured two very different AD 79 palaeosols to find the reservoir ages shown in Table 2. The three fractions of the organic-rich palaeosol from the necropolis had unusually similar and relatively young reservoir ages at burial, which is to be expected for a soil under cultivation and receiving high organic input²³. The more relevant, less carbonaceous, AD 79 palaeosol at the Ottaviano quarry followed trends in genetic soil ages and indicated that little rejuvenation had occurred after burial. The humin fraction (RIDDL-1405) was exceptionally old, but such refractory carbon is difficult to characterize. The reservoir age of the hydrolysate fraction was small or null, but had a large uncertainty due to the small sample size. The humic acid reservoir, often used in dating, was 460 years old at burial. To obtain an age of the Avellino event from soil fractions, we subtracted

these derived reservoir ages: 450 ± 150 ^{14}C yr for the humic acids, and 200 ± 200 yr for the hydrolysate fraction. These reservoir-corrected ages agreed with the charcoal dates, within the large uncertainties imposed by the reservoir uncertainties.

The ages of the two charcoals best associated with the Avellino eruption and the four corrected soil ages averaged ($1/\sigma^2$ weighted) to $3,360 \pm 40$ yr BP ($\chi^2/\nu = 1.28$; $\nu = 5$). The calibrated date corresponding to this ^{14}C age (at 1σ uncertainty) is in the ranges 1617-1703 cal BC ($P = 0.84$) and 1716-1735 cal BC ($P = 0.16$), using the standard bi-decadal calibration program²⁴. Bronze-age ceramics (seventeenth-eighteenth century BC) beneath the Avellino pumice²⁵ support this result, rather than the previous nineteenth-twentieth century BC date. This change of several centuries from previous age estimates for Avellino reduces the calculated repose time before the AD 79 eruption. As Vesuvius may currently be in a pre-plinian phase²⁶, this decrease in the estimated time between the last two major plinian eruptions is significant for risk assessment.

Having found one erstwhile nineteenth century BC event to be much younger, we re-evaluated the data for the eruptions of Mount St Helens Y_n ($46^\circ 38' \text{N}$, $119^\circ 46' \text{W}$) and Aniakhak II ($56^\circ 56' \text{N}$, $158^\circ 37' \text{W}$). The initial nineteenth century interpretations of Y_n ^{27,28} arose from use of an earlier, less accurate radiocarbon calibration. Current data for Y_n are summarized in Table 3. Peat dates at well defined tephra layers yield a weighted average of $3,420 \pm 50$ yr BP. As with an uncorrected soil date, this must be an upper limit. A thickness of 2 cm of peat represents ≥ 300 years of growth²⁹; tephra slows rejuvenation of underlying peat, and a later eruption covered ≥ 260 -year-old organics³⁰. Ages of wood and charcoal samples average ($1/\sigma^2$ weighted) to $3,410 \pm 40$ yr BP. Size requirements for decay counting imply that an old-wood correction of 50 ± 50 yr is reasonable, yielding an age of $3,360 \pm 65$ BP (1,559-1,536 cal BC, $P = 0.11$; 1,601-1,742 cal BC, $P = 0.89$). Aniakhak II is dated from six peat samples to a weighted average of $3,560 \pm 40$ yr BP without reservoir corrections³¹. Two large buried charcoals (Table 3) average to $3,390 \pm 90$ yr BP after a 50 ± 50 year old-wood correction, and carbonized twigs at the ash-flow base are $3,350 \pm 200$ BP without corrections, yielding an estimate of $3,380 \pm 90$ yr BP (1530-1563 cal BC, $P = 0.12$; 1598-1773 cal BC, $P = 0.80$; 1845-1867 cal BC, $P = 0.08$). Thus, Avellino, St Helens Y_n , and Aniakhak II all have ^{14}C ages and uncertainties that make them compatible with the preferred ^{14}C ages for the eruption of Thera^{14,32-34}.

An acidity layer in a Greenland ice core¹⁴, frost damage to

TABLE 2 AD 79 eruption materials and radiocarbon dates

Identification	Lab. No. RIDDL-	$\delta^{13}\text{C}^*$ (‰)	Age† (yr BP)	Error‡ (±)	Reservoir/apparent§ age at burial ($\pm 1\sigma$)	
Nocera gate necropolis, Pompei						
Small charcoal piece (295 μg)	1346	(-25)	1,940	80	13	(-50, 115)
Palaeosol						
hydrolysate	1347	(-24)	2,210	70	290	80
humics	1348	-24.5	2,255	70	335	80
humin	1349	-24.7	2,255	75	335	80
Ottaviano quarry						
Palaeosol						
hydrolysate	1403	(-24)	2,050	180	130	190
humics	1404	(-25)	2,380	95	460	100
humin	1405	(-25)	4,950	110	2,670	120
Herculaeneum waterfront						
Partially carbonized door lintel (25 $\times$ 30 cm cross-section)						
Uncharred wood	1332	-23.9	2,000	150		
	1333	-23.9	1,980	60		
Charred wood	1334	-23.7	2,040	45		
	1335	-23.5	1,970	55	74	(60, 130)
Large carbonized beam	1336	-24.5	2,070	65		
	1337	(-25)	2,060	80	145	(85, 205)

* Isotope fractionation corrections were made from $\delta^{13}\text{C}$ measurements on the larger samples. Assumed values for other samples are in parentheses. $\delta^{13}\text{C}$ measurements are referenced to PDB standard in units per mil (‰).

† All dates are reported using the international conventions of Stuiver and Polach³⁹.

‡ Uncertainty is the standard deviation of multiple (≥ 3) measurements expressed as 1σ .

§ Apparent age for wood and charcoal at burial was calculated as the difference between AD 79 and the calibrated ^{14}C date. Multiple samples of the same material were averaged before calibration. The uncertainty in this age is given as the calibrated age range at 1σ . The reservoir age of the soil samples at burial was calculated as the difference between a ^{14}C age of 1,920 yr BP (corresponding to AD 79 using the 100-year averaged calibration of Stuiver and Pearson⁴⁰) and the measured ^{14}C age for soil fraction samples. Uncertainties given as 1σ .

tree rings in western North America¹² and narrow growth rings in Irish oak trees¹³ have been ascribed to the Thera eruption. As there is no *a priori* reason to associate distant proxy records with specific contemporaneous volcanic eruptions³⁵, there may be contributions from all, one or none of these volcanoes in these records. It is even possible that the markers at 1645 BC in the ice core and at 1627 BC in the two tree-ring series recorded separate events. Indeed, the frequency of large eruptions is so high²⁸ that other candidates may exist which have not yet been identified.

If temporal data alone cannot definitively link specific eruptions with distant climate records, other geochemical analyses must be used. For example, the ice-core acidity layer contained high levels of sulphate¹⁴, but recent petrological calculations of the sulphur emissions from Thera account for only 3–6% of the amount expected from the concentration of acid in the ice layer (ref. 36 and H. Sigurdsson and S. Carey, manuscript in preparation). We roughly estimated the sulphur emissions from the

other eruptions in a similar manner⁷. If aerosols from Avellino approximated that of the very similar AD 79 eruption³⁷, they would have been predominately sulphurous and three times more abundant than those from the larger Thera eruption. Mount St Helens Y_n may have yielded only half as much sulphate as Thera, but Aniakchak II could have produced sulphur emissions four times greater than Thera's. Given the uncertainties in calculating aerosol transport from these different locations to Southern Greenland, Thera is no more likely than any of these others to have produced the observed acid layer. Sulphur isotope ratios or micro-shard comparison may yet identify the source, as long as all possible candidates have been identified. Unfortunately, it is unlikely that such geochemical comparisons can be applied to the tree rings.

Comparisons of diverse records of past phenomena require that the chronological data be examined carefully. Accurate dating requires a direct association between the dated material and the event, as well as a precise measurement. Radiocarbon dates for eruptions have often been obtained from palaeosol, peat and other organic samples without reservoir age corrections. However, ^{14}C dating by AMS now allows greater selectivity of samples. Twigs, seeds³⁸ and even pollen³⁹, which can be more directly associated with an event, can now be dated. □

TABLE 3 Mount St Helens Y_n and Aniakchak II radiocarbon dates

Identification	Lab. No.	Age (yr BP)	Error $\pm$	Reference
Y_n tephra, Alberta, Canada				
Charcoal below Y_n	Beta-4676	3,370	100	41
Peat around Y_n	AECV-451-C	3,350	100	42†
Peat around Y_n	WIS-343	3,550	65	43
Y_n tephra, British Columbia, Canada				
Peat above Y_n	Beta-13558	3,140	70	29
Peat below Y_n	Beta-13559	3,680	80	29
Charcoal below Y_n	GSC-345	3,410	65*	44
Peat below Y_n	GSC-298	3,390	65*	44
Wood above Y_n	GSC-1461	3,430	70*	44
Y_n ashfall, Washington, USA				
Charcoal above Y_n	W-2549	3,350	260	27
Charcoal below Y_n	W-1752	3,510	240	27
Aniakchak II ashflow, Alaska, USA				
Charcoal twigs at base	W-3125	3,350	200	31
Charcoal log at in flow	I-14221	3,410	90	31
Charcoal in pumice	I-14226	3,520	140	31

* Uncertainty reduced to 1σ from the reported 2σ to conform to convention³⁹.

† Reference contains other dates used to project age of discontinuous tephra. Only the date taken directly around the tephra is used here.

Received 21 September 1989; accepted 16 February 1990.

- Barbieri, F., Bizouard, H., Clocchiatti, R., Metrich, N., Santacroce, R. & Sbrana, A. *Bull. volcanol.* **44**, 295–315 (1981).
- Sigurdsson, H., Carey, S., Cornell, W. & Pescatore, T. *Nat. Geol. Res.* **1**, 332–387 (1985).
- Carey, S. & Sigurdsson, H. *Geol. Soc. Am. Bull.* **99**, 303–314 (1987).
- Carey, S. & Sigurdsson, H. *Bull. volcanol.* **51**, 28–40 (1989).
- Rampino, M. R. & Self, S. *Quat. Res.* **18**, 127–143 (1982).
- Rampino, M. R., Stothers, R. B. & Self, S. *Nature* **313**, 272 (1985).
- Devine, J. D., Sigurdsson, H., Davis, A. N. & Self, S. *J. geophys. Res.* **89**, 6309–6325 (1984).
- Lirer, L., Pescatore, T., Booth, B. & Walker, G. P. L. *Geol. Soc. Am. Bull.* **84**, 759–772 (1973).
- Alessio, M., Bella, F., Improta, S., Belluomini, G., Cortesi, C. & Turi, B. *Radiocarbon* **13**, 395–411 (1971).
- Alessio, M. et al. *Radiocarbon* **16**, 358–367 (1974).
- Delibrias, G., Guillier, M.-T. & Labeyrie, J. *Radiocarbon* **28**, 9–68 (1987).
- LaMarche, V. C. Jr. & Hirschboeck, K. K. *Nature* **307**, 121–126 (1984).
- Baillie, M. G. L. & Munro, M. A. R. *Nature* **332**, 344–346 (1988).
- Hammer, C. U., Clausen, H. B., Friedrich, W. L. & Tauber, H. *Nature* **328**, 517–519 (1987).
- Geyh, M. A. & Roeschmann, G. *Radiocarbon* **25**, 409–416 (1983).
- Delibrias, G., DiPaola, G. M., Rosi, M. & Santacroce, R. *R. Soc. Ital. Miner. Petrol.* **35**, 411–438 (1979).
- Radice, B. (ed.) *The Letters of the Younger Pliny* (Penguin, New York, 1983).
- Sakai, C., Sakagami, K., Hamada, R. & Kurobe, T. *Soil Sci. Plant Nutr.* **28**, 37–48 (1982).

19. Vogel, J. S., Nelson, D. E. & Southon, J. R. *Radiocarbon* **31**, 145–149 (1989).
20. Campbell, C. A., Paul, E. A., Rennie, D. A. & McCallum, K. J. *Soil Sci.* **104**, 217–224 (1967).
21. Goh, K. M., Stout, J. D. & O'Brien, B. J. *N.Z. J. Sci.* **27**, 69–72 (1984).
22. Trumbore, S., Vogel, J. S. & Southon, J. *Radiocarbon* (in the press).
23. Campbell, C. A., Paul, E. A., Rennie, D. A. & McCallum, K. J. *Soil Sci.* **104**, 81–85 (1967).
24. Stuiver, M. & Reimer, P. J. *Radiocarbon* **28**, 1022–1030 (1986).
25. Albore Livadie, C. *Atti Acc. Naz. L. Not. S. Ant.* **8**, 59–101 (1981).
26. Santacroce, R. J. *Volcanol. geotherm. Res.* **17**, 237–248 (1983).
27. Mullineux, D. R., Hyde, J. H. & Rubin, M. J. *Res. US geol. Surv.* **3**, 329–335 (1975).
28. Simkin, T., Siebert, L., McClelland, L., Bridge, D., Newhall, C. & Latter, J. H. *Volcanoes of the World* (Hutchinson, Stroudsburg, 1981).
29. Luckman, B. H., Kearney, M. S., King, R. H. & Beaudoin, A. B. *Can. J. Earth Sci.* **23**, 734–736 (1986).
30. Hoblitt, R. P., Crandell, D. R. & Mullineux, D. R. *Geology* **8**, 555–559 (1980).
31. Miller, T. P. & Smith, R. L. *Geology* **15**, 434–438 (1987).
32. Cadogan, G. *Nature* **328**, 473 (1987).
33. Manning, S. W. *Nature* **332**, 401 (1988).
34. Hammer, C. U., Clausen, H. B., Friedrich, W. L. & Tauber, H. *Nature* **332**, 401 (1988).
35. Pyle, D. M. *Archaeometry* **31**, 88–91 (1989).
36. Sigurdsson, H., Devine, J. D. & Davis, A. N. *Jökull* **35**, 1–8 (1985).
37. Cornell, W. thesis, University of Rhode Island (1987).
38. Peteet, D. M., Vogel, J. S., Nelson, D. E., Southon, J. R., Nickman, R. J. & Heusser, L. E. *Quat. Res.* (in the press).
39. Brown, T. A., Nelson, D. E., Mathewes, R. W., Vogel, J. S. & Southon, J. R. *Quat. Res.* **32**, 205–212 (1989).
40. Stuiver, M. & Polach, H. *Radiocarbon* **19**, 355–363 (1977).
41. Stuiver, M. & Pearson, G. W. *Radiocarbon* **28**, 805–838 (1986).
42. King, R. H. *Correlation of Quaternary Chronologies* (ed. Mahaney, W. C.) 243–258 (Geobooks, Norwich, 1984).
43. Zolai, S. C. *Can. J. Earth Sci.* **26**, 207–214 (1989).
44. Westgate, J. A. *Can. J. Earth Sci.* **14**, 2593–2600 (1977).
45. Fulton, R. J. *Geol. Surv. Can. Pap.* **71–37**, 19–21 (1971).

ACKNOWLEDGEMENTS. We thank W. Jashemski, S. Carey and P. Hatcher for samples, and H. Sigurdsson, J. Foss and R. McNeely for discussions. B. Nielsen made the $\delta^{13}\text{C}$ determinations. ^{14}C measurements were made using the Tandem Accelerator at McMaster University supported by NSERC (Canada) and RIDD.

ESR dating evidence for early modern humans at Border Cave in South Africa

Rainer Grün*, Peter B. Beaumont† & Christopher B. Stringer‡

* Subdepartment of Quaternary Research, Cambridge University, Free School Lane, Cambridge CB2 3RS, UK

† Department of Archaeology, McGregor Museum, PO Box 316, Kimberley 8300, South Africa

‡ Department of Palaeontology, Natural History Museum, Cromwell Road, London SW7 5BD, UK

THE archaeological and hominid site of Border Cave (KwaZulu, South Africa) has a stratigraphic sequence covering the Middle and Later Stone Ages (MSA and LSA)^{1–10}. It has been proposed that four hominid specimens recovered there (BC1 and BC2 of uncertain provenance, and BC3 and BC5 recovered from MSA layers) represent very early examples of anatomically modern humans, supporting an early late-Pleistocene appearance of modern *Homo sapiens* in Africa. This early appearance, however, has been questioned, largely because of doubts about the stratigraphic positions associated with the specimens and because of the lack of a reliable chronology for the stratigraphic sequence. We now report on the first comprehensive radiometric dating analysis of Border Cave, using electron spin resonance (ESR) on teeth within sediment layers. BC3 is likely to be ~70–80 kyr old, and BC5, 50–65 kyr old. BC1 and BC2 are almost certainly less than 90 kyr old. These results, although younger than some age estimates, support the early occurrence of anatomically modern humans at Border Cave. In addition, our results suggest that the Howiesons Poort lithic industry (~45–75 kyr) and the MSA-LSA transition (~35 kyr) are younger than often believed.

The stratigraphic sequence of Border Cave reaches a cumulative depth of 4 m, and is characterized by an alternating series of units termed 'Brown Sands' (BS) and 'White Ashes' (WA)^{1–4}. The entire Pleistocene sequence of Border Cave has not previously been dated by any one method: the dating of the lower sequence of deposits by extrapolations from radiocarbon age determinations of the upper parts of the sequence^{3,4} has been

TABLE 1 Origin and species of teeth

Unit	Sample no.	Species
1BS.LR1	531	<i>Bovini</i> , upper molar
	644	<i>Syncerus caffer</i> , lower premolar
	646	<i>Hippotragus</i> sp., lower premolar
1WA.UP	645	<i>Damaliscus dorcas</i> , lower molar
	1WA	<i>Bovini</i> , upper premolar
	540	<i>Potamochoerus porcus</i> , lower molar
2BS.UP	532	<i>Equus</i> , lower premolar
	533	<i>Equus</i> , lower premolar
	537	<i>Syncerus caffer</i> , upper molar
2BS.LRC	529	<i>Equus burchelli</i> , upper molar
	541	<i>Equus</i> , lower premolar
	535	<i>Bovidae</i> , molar fragments
3BS.1	608	<i>Equus</i> , molar fragments
	607	Fragments (ND)
	607	Fragments (ND)
3BS.2	536	<i>Bovidae</i> , molar fragments
	609	Fragments (ND)
	601	Fragments (ND)
3BS.3	534	<i>Damaliscus dorcas</i> , upper molar
	538	<i>Bovini</i> , lower molar
	647	Fragments (ND)
3WA	602	<i>Syncerus caffer</i> , upper premolar
	603	Fragments (ND)
	604	Fragments (ND)
1RGSB	605	<i>Hippotragus niger</i> , upper molar
	539	<i>Phacochoerus aethiopicus</i> , molar
	539	

ND, Not determined.

criticized as unreliable^{11,12}. It has therefore not been possible to independently test proposed palaeo-environmental 'matches' with the oxygen isotope record derived from lithostratigraphic and faunal studies^{3,4,6}.

Radiocarbon dating analysis, mainly of charcoal, has given ages from 0.65 kyr to 28.5 kyr for level 1BS.UP (below iron-age layers), 33 kyr to 38.6 kyr for level 1BS.LR, 33 kyr to 45 kyr for level 1WA, and >41 kyr to >49.1 kyr for level 2BS.UP^{1,2}.

Specimen BC1, an adult partial cranial vault, and possibly associated postcranial fragments, and BC2, a partial adult mandible, were recovered out of stratigraphic context in 1941–1942. Some other postcranial fragments, which could be associated with BC1, have been found recently¹³. Because the surface of unit 4WA was apparently intact in 1942, these specimens could, in principle, have derived from any overlying level, but matrix on the BC1 cranial fragments appeared most similar to that from the base of unit 4BS^{1,2}. Specimen BC3 is a partial skeleton of a young infant, recovered in 1941, apparently from a shallow grave cut into unit 4BS, and contemporary with the level. Specimen BC5 is a nearly complete adult mandible, containing four teeth, recovered from a shallow pit in level 3WA.LR in 1974. Most researchers attribute the Border Cave hominid specimens to modern *Homo sapiens*, although they are not agreed as to the precise affinities of the specimens^{9,11,13–22}. Nevertheless, it has been questioned whether the specimens (in particular BC1) represent modern *H. sapiens*^{20,23}, and whether, because of their relatively good preservation, they are contemporaneous with MSA levels²⁴.

To establish an absolute chronology for Border Cave, we made ESR measurements on 25 tooth samples. The position of the teeth within the sediment layers and the species from which they came are given in Table 1. The principles of ESR dating have been described recently^{25,26}. We analysed two to four enamel pieces from each tooth. The accumulated dose was determined by the additive dose method using exponential curve fitting and a 'jack-knifing' procedure for error calculation²⁷. The uranium concentrations of enamel and dentine were measured by neutron activation analysis. The external β -dose rate was deduced from the neutron activation analysis of the sediment ($\leq 300\ \mu\text{m}$) adjacent to the teeth. The concentration of radioactive isotopes were converted into dose rates using tables by Nambi and Aitken²⁸ and beta attenuation factors from Grün²⁹.

538

NATURE · VOL 344 · 5 APRIL 1990

Abbreviations: EN, enamel; DE, dentine; SED, sediment; β -, β dose rate; γ -, γ dose rate; int., internal dose rate.
 * The values for thickness of enamel and the layers removed from both sides (to eliminate the volume irradiated by external α -rays) were used to calculate the β -attenuation factors²⁹.
 † Enamel layer was separated from the inside of the tooth between two dentine layers. No β -irradiation from the sediment.
 ‡ AD, accumulated doses. ADs and uncertainties were calculated with the computer program FITT²⁷. The uncertainty also includes a systematic error of 5% due to the calibration of the γ -source.
 § The uncertainty of the external γ -dose rate arises from a systematic calibration error of 5%.
 || The laboratory uncertainty of neutron activation analysis is negligible for dose rate calculation.
 ¶ The sample showed a thin coating of cement. The external β -dose rate was corrected accordingly.

The external γ -dose rate was measured *in situ* with a portable gamma spectrometer. The analytical values and ESR results are given in Table 2.

The present-day U concentration of enamel and dentine is usually near the detection limit of 10 p.p.b. (Table 2). Normally, enamel and dentine tend to accumulate uranium over time, which can cause large uncertainty in age estimates^{25,26,30}. But, uniquely, the U concentrations in nearly all the Border Cave samples do not exceed those found in modern teeth, indicating that the ESR dates have not been influenced by any U mobilization. This implies that the cave was completely dry throughout most of its depositional history, as it is now.

The ESR results show internal stratigraphic consistency (see Fig. 1). For unit 1BS, the ESR results agree fairly well with radiocarbon dates (see above). But for units 1WA and 2BS, the oldest ¹⁴C dates are about 30% older than the ESR estimates. It is unlikely that this divergence is attributable to a systematic error such as source calibration or calibration of the gamma spectrometer.

We observed two main outliers: samples 536B and 603C (see Fig. 1). Both were small enamel pieces, and it cannot be excluded that they were introduced into the sediment at a later date. Repeated analyses on single teeth do not show such large scattering. The only major reversal of dates is represented by sample 539 which is apparently younger than samples 604 and 605 in the covering layers. The concentration of radioactive isotopes in sediment adjacent to this sample is far lower than that in the

rest of the profile, and the external γ -dose rate at the precise site of the sample could be substantially lower than the measured value of 1,390 $\mu\text{Gy yr}^{-1}$ for layer 5BS. This would lead to an older date.

If the stratigraphic position from which BC3 was recovered has been correctly identified, then this specimen is likely to be 70–80 kyr old, based on ages for the 4BS and 1RGS layers, and <90 kyr old, based on the mean age determined for the uppermost 4WA.1. If specimens BC1 and BC2 were derived from the levels immediately overlying level 4WA^{1,2}, their ages should be similar to that of BC3. We hope, however, that direct radiocarbon-accelerator dating of BC1, BC2 and BC5 in progress at Oxford will help to establish the antiquity of these specimens. BC5 is likely to be about 50–65 kyr old, based on mean age determinations for layer 3WA and the overlying lower 3BS level.

The Howiesons Poort (also known as Epi-Pietersburg or MSA2)² lithic industry is interesting in that it shows several 'advanced' aspects that seem to anticipate those of the LSA and Upper Palaeolithic elsewhere^{1,2,7–10}. Our results place the Howiesons Poort layers in a wider and younger time range (about 45–75 kyr ago) than often believed⁹, consistent with ESR results from Klasies River Mouth Caves³¹.

ESR dating therefore provides support for an early presence of anatomically modern humans in southern Africa, although the probable ages of the BC hominids (and of the Howiesons Poort and MSA3) are younger than the greatest ages previously inferred. Although links with present African populations are difficult to identify, genetic data now seem to indicate that regional differentiation of African populations had begun by this time, and one analysis³² suggests that Khoisan origins predate the age we infer for the BC hominids. □

Received 23 November 1989; accepted 2 February 1990.

1. Beaumont, P. B., de Villiers, H. & Vogel, J. C. S. *Afr. J. Sci.* **74**, 409–419 (1978).
2. Beaumont, P. B. *Palaeont. afr.* **23**, 21–33 (1980).
3. Butzer, K. W., Beaumont, P. W. & Vogel, J. C. *J. arch. Sci.* **5**, 317–341 (1978).
4. Klein, R. G. (ed.) *Southern African Prehistory and Palaeoenvironments* (Balkema, Rotterdam, 1984).
5. Klein, R. G. S. *Afr. archaeol. Bull.* **32**, 14–27 (1977).
6. Avery, D. M. *J. arch. Sci.* **9**, 187–204 (1982).
7. Sampson, C. G. *The Stone Age Archaeology of Southern Africa* (Academic, New York, 1974).
8. Singer, R. & Wymer, J. *The Middle Stone Age at Klasies River Mouth in South Africa* (University of Chicago Press, Chicago, 1982).
9. Mellars, P. & Stringer, C. B. (eds) *The Human Revolution: Behavioural and Biological Perspectives on the Origins of Modern Humans* (Edinburgh University Press, 1989).
10. Mellars, P. *Curr. Anthropol.* **30**, 349–385 (1989).
11. Smith, F. H. Z. *morph. Anthropol.* **75**, 197–222 (1985).
12. Parkington, J. in *The Human Revolution: Behavioural and Biological Perspectives on the Origin of Modern Humans Vol. 2* (ed. Mellars, P.) (Edinburgh University Press) (in the press).
13. Morris, A. in *Continuity or Replacement? Controversies in the Evolution of Homo sapiens* (eds Bräuer, G. & Smith, F. H.) (Balkema, Rotterdam) (in the press).
14. De Villiers, H. *Ann. Transv. Mus.* **28**, 229–256 (1973).
15. De Villiers, H. S. *Afr. J. Sci.* **72**, 212–215 (1976).
16. De Villiers, H. & Fatti, L. P. S. *Afr. J. Sci.* **78**, 321–332 (1982).
17. Fatti, L. P. in *Variation, Culture and Evolution in African Populations* (eds Finger, R. & Lundy, J. K.) 27–34 (Witwatersrand University Press, Johannesburg, 1986).
18. Rightmire, G. P. *Current Anthropol.* **20**, 23–26 (1979).
19. Rightmire, G. P. *Current Anthropol.* **22**, 199–200 (1981).
20. van Vark, G. N. & Howells, W. W. *Multivariate Statistical Methods in Physical Anthropology* (Reidel, Dordrecht, 1984).
21. Bräuer, G. in *The Origins of Modern Humans: A World Survey of Fossil Evidence* (eds Smith, F. H. & Spencer, F.) 327–410 (Liss, New York, 1984).
22. Habgood, P. J. S. *Afr. archaeol. Bull.* **44**, 17–22 (1989).
23. van Vark, G. N., Bilsborough, A. & Dijkema, J. *Anthropol. Prehist.* **100**, 43–56 (1989).
24. Klein, R. G. A. *Rev. Anthropol.* **12**, 25–48 (1983).
25. Grün, R. *Die ESR-Alterbestimmungsmethode* (Springer, Heidelberg, 1989).
26. Grün, R. *Quat. Int.* **1**, 65–109 (1989).
27. Grün, R. & Macdonald, P. D. M. *Applied Radiation and Isotopes* **40**, 1077–1080 (1989).
28. Nambi, K. S. V. & Aitken, M. J. *Archaeometry* **28**, 202–205 (1986).
29. Grün, R. *Ancient TL* **4**, 1–8 (1986).
30. Grün, R., Schwarcz, H. P. & Zymela, S. *Can. J. Earth Sci.* **24**, 1022–1037 (1987).
31. Grün, R., Shackleton, N. J. & Deacon, H. *Curr. Anthropol.* (in the press).
32. Vigilant, L., Pennington, R., Harpending, H., Kocher, T. & Wilson, A. *Proc. natn Acad. Sci. U.S.A.* **86**, 9350–9354 (1989).
33. Martinson, D. G. *et al. Quat. Res.* **27**, 1–29 (1987).

ACKNOWLEDGEMENTS. We thank A. Gentry, and E. A. Voigt for determining the species of the teeth, H. P. Schwarcz for the gamma spectrometer, J. Vogel for organizing the field trip to Border Cave, P. Clay for access to the γ -source of Imperial College, London, L. Sutcliffe for the use of the ESR spectrometer, A. Wieser for calibrating the γ -source, and A. Krebber for her hospitality. This study was supported by NERC and SERC (N.S.). The 1987 excavations at Border Cave were funded by the Anglo-American and De Beers Chairmen's Fund (P.B.B.). R.G. was supported by the D. M. MacDonald Fund to the Department of Archaeology, Cambridge, UK.

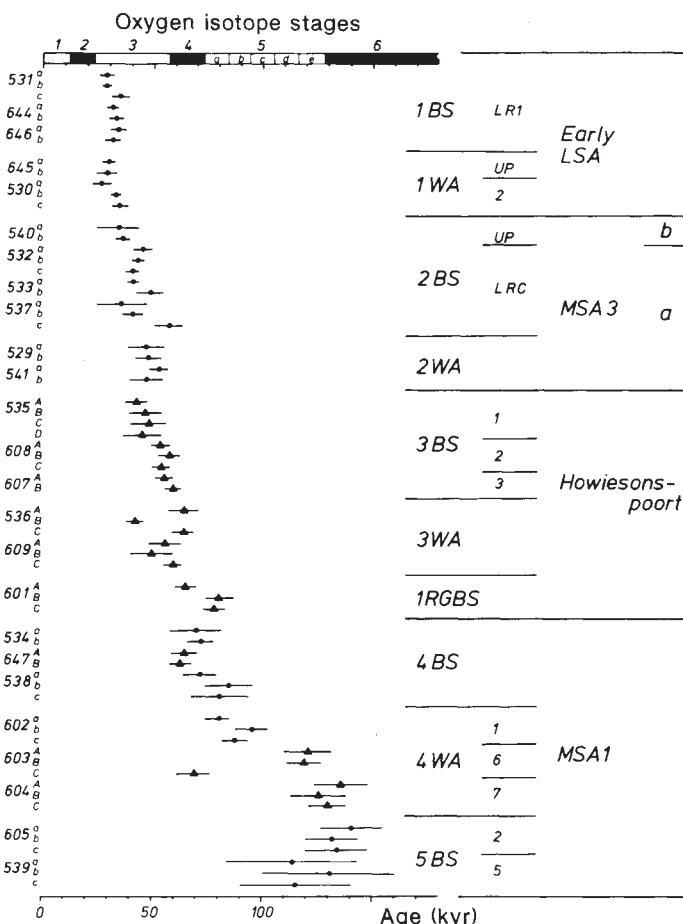


FIG. 1 ESR estimates on tooth samples from Border Cave. ●, Mean ages with repeated analyses on one tooth; ▲, mean ages with analyses on separate tooth fragments. Bar, 1 s.d. error for each individual estimate. The stratigraphic provenance (1BS to 5BS) of the samples is given, as well as their archaeological association, using the nomenclature of Beaumont². The Howiesons Poort was also classified as MSA2 (ref. 2). Oxygen isotope stages were taken from Martinson *et al.*³³. RGS, Rubby Grey-Brown Sand.

Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3

L. M. Brzustowicz*, T. Lehner†, L. H. Castilla*, G. K. Penchaszadeh*, K. C. Wilhelmsen*, R. Daniels‡, K. E. Davies‡, M. Leppert§, F. Ziter||, D. Wood¶, V. Dubowitz**, K. Zerres††, I. Hausmanowa-Petrusewicz‡‡, J. Ott†, T. L. Munsat§§ & T. C. Gilliam*|||

* Departments of Psychiatry and Neurology, and † Department of Psychiatry, Genetics and Development, Columbia University, and New York State Psychiatric Institute, New York, New York 10032, USA

‡ Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

§ Howard Hughes Medical Institute and Department of Human Genetics, and

|| Departments of Pediatrics and Neurology, University of Utah Medical Center, Salt Lake City, Utah 84132, USA

¶ Muscular Dystrophy Association of America, New York, New York 10019, USA

** Royal Postgraduate Medical School, Hammersmith Hospital, London, W12, UK

†† Institute for Human Genetics, University of Bonn, FRG

‡‡ Akademia Medyczna, Klinika Neurologiczna, Warszawa, Poland

§§ Department of Neurology, Tufts University-New England Medical Center, Boston, Massachusetts 02111, USA

Present address: Odyssey Biomedical Corporation, 885 Second Avenue, New York, New York 10617, USA

||| To whom correspondence should be addressed

SPINAL muscular atrophy (SMA) describes a group of heritable degenerative diseases that selectively affect the α -motor neuron. Childhood-onset SMAs rank second in frequency to cystic fibrosis among autosomal recessive disorders, and are the leading cause of heritable infant mortality. Predictions that genetic heterogeneity underlies the differences between types of SMA, together with the aggressive nature of the most-severe infantile form, make linkage analysis of SMA potentially complex. We have now analysed 13 clinically heterogeneous SMA families. We find that 'chronic' childhood-onset SMA (including intermediate SMA or SMA type II, and Kugelberg-Welander or SMA type III) is genetically homogeneous, mapping to chromosomal region 5q11.2-13.3.

The nosology of childhood SMA is not clear. Most cases are characterized by symmetrical weakness and atrophy of limb muscles, degeneration of anterior horn cells, and evidence of

denervation with normal conduction times¹⁻⁴. The acute form of SMA (Werdnig-Hoffmann, infantile SMA, severe SMA, type I) with onset in infancy is often fatal in the first year and usually by four years, and seems to constitute a clinically homogeneous subgroup⁵. Patients who follow a more chronic course form a clinically heterogeneous subgroup described alternatively as 'chronic childhood SMA' or SMA type II (intermediate SMA) and SMA type III (Kugelberg-Welander, mild SMA)⁴. Age of onset varies (6 months to 17 years) and symptoms range from never being able to walk and possible death in adolescence, to only mild muscle weakness. Childhood-onset SMA is recessively inherited, whereas adult-onset (17-55 years) SMA is transmitted in either a recessive or dominant pattern⁶. An X chromosome-linked form of SMA has also been reported⁷. It is not known whether the clinical variation among childhood SMA cases reflects the variable expressivity of a single gene defect, or the expression of variable, possibly unlinked, disease alleles with clinically overlapping phenotypes^{8,9}.

The incidence of acute SMA is 1 in 20,000 live births¹⁰, with a carrier frequency of 1 in 60 to 1 in 80, and an estimated gene frequency of 0.006. The chronic forms affect a similar number—1 in 24,000 (refs 11, 12). SMA carriers are clinically normal, and have normal electromyographs and muscle biopsies. Although concordance of severity in a family is usual, cases of varying severity in an affected sibship have occasionally been reported^{2,3,8}.

We studied seven families diagnosed with chronic SMA, and six families diagnosed with acute SMA (pedigrees available from the authors on request). Age of onset in chronic families was 2-10 years, and symptoms ranged from death in adolescence to only minor impairment of function, with affected individuals meeting criteria for either the intermediate or mild forms of childhood-onset SMA (types II and III)⁴. Age of onset in acute cases ranged from birth to 6 months, and age at death ranged from 8 months to 2 years. One child has survived to 5 years of age with the aid of a respirator. Because of the aggressive nature of acute SMA, it is difficult to locate families with many living affected children. All 13 families meet diagnostic criteria for SMA as determined by the ascertainment team headed by one of us (T.L.M.).

To identify markers for use in linkage analysis, we initiated a blind search through the genomes of our three most-informative chronic SMA families. Linkage was detected after testing a total of 115 DNA markers, so that a maximum log likelihood ratio (lod score) of three or more is appropriate for declaring linkage^{13,14}. Multipoint lod scores were calculated as $\log_{10} [L(x)/L(\infty)]$, where x is the multipoint likelihood with respect to the location x of the disease on the fixed map of markers. The results of genetic linkage analysis are shown in Fig. 1 and Table 1. Figure 1 shows multipoint linkage analysis between eight DNA markers and the disease locus in chronic

TABLE 1 Pairwise lod scores for chronic and acute SMA

Locus	Probe	Recombination fraction								
		0	0.02	0.05	0.1	0.15	0.2	0.25	0.3	0.35
Chronic SMA										
D5S6	LM4	−∞	8.43	7.99	6.90	5.71	4.52	3.37	2.32	1.43
D5S39	p105-153Ra	−∞	5.58	5.56	4.83	3.93	3.02	2.19	1.48	0.91
D5S78	p105-798Rb	−∞	1.01	1.46	1.60	1.50	1.32	1.09	0.84	0.58
D5S37	π 227	−∞	−0.02	0.60	0.86	0.87	0.78	0.63	0.46	0.30
Acute SMA										
D5S6	LM4	−∞	−1.50	−0.50	−0.00	0.14	0.19	0.17	0.13	0.08
D5S39	p105-153Ra	−∞	1.01	1.46	1.60	1.50	1.32	1.09	0.84	0.58
D5S78	p105-798Rb	−∞	1.71	1.50	1.17	0.87	0.62	0.42	0.26	0.14

Two-point lod scores were evaluated using the MLINK program of the LINKAGE package at selected male recombination fractions²⁰. Chronic SMA refers to cases which meet diagnostic criteria for intermediate (SMA type II) and mild (Kugelberg-Welander, SMA type III) forms⁴. Acute SMA refers to cases which meet diagnostic criteria for SMA type I (Werdnig-Hoffmann, infantile SMA, severe SMA).

families (solid line) and acute families (dotted line). The peak multipoint lod score for chronic SMA is 9.03, and the peak lod score for acute SMA is 2.02. Pairwise lod scores for chronic and acute SMA families versus four markers located in the middle of the linkage region are shown in Table 1. The maximum two-point lod score for chronic families is 8.43 at a recombination fraction of 2% with marker D5S6, and 1.71 for acute families at a recombination fraction of 2% with marker D5S78.

Application of the HOMOG program¹³ to the multipoint lod scores of the families with chronic SMA gave no evidence for heterogeneity among these families. Although the power of homogeneity tests can be lower in recessive families than in larger families with dominant diseases, the absence of evidence for heterogeneity led us to adopt the most parsimonious solution of assuming homogeneity. The confidence interval for the location of the gene for chronic SMA is 11 centimorgans (cM) wide and spans a region 2 cM proximal of locus D5S6 to a point 4 cM proximal of locus D5S78 (note arrows in Fig. 1). For families with acute SMA, the maximum lod score of 2.02 indicates that a gene responsible for this disease maps to the same general area. The best estimate for the location of the acute SMA locus is 15 cM distal to the estimated position of the locus for chronic SMA.

Our data indicate that clinically heterogeneous forms of chronic childhood SMA (type II or intermediate form and type III or Kugelberg-Welander or mild form) map to a single locus on chromosome 5q. The chronic forms of childhood-onset SMA, therefore, are likely to occur as the result of allelic heterogeneity, similar to the case for Duchenne- and Becker-type dystrophies¹⁵. It is interesting that our data indicate that acute childhood SMA

(type I or Werdnig-Hoffmann or infantile SMA or severe SMA) map to the same, or a closely linked, locus on 5q. Other informative acute families must be analysed to confirm the linkage of this form of SMA and to evaluate the associated map location relative to that of chronic SMA. Also, other chronic families must be analysed to further assess the possible occurrence of nonallelic heterogeneity. It will be interesting to determine whether adult-onset and dominantly inherited cases of SMA similarly map to chromosome 5q. The gene encoding hexosaminidase B maps between markers D5S39 and D5S78 (refs 16, 17). Deficiencies in both the α - and β -subunit of this enzyme have been associated with chronic cases of SMA^{18,19}. We are investigating whether this gene is a candidate for an SMA mutation. □

Received 11 January; accepted 16 February 1990.

1. Pearn, J. H. *Adv. Neurol.* **36**, 121-130 (1982).
2. Emery, A. E. H., Davie, A. M., Holloway, S. & Skinner, R. J. *Neurol. Sci.* **30**, 375-384 (1976).
3. Munsat, T. L., Woods, R., Fowler, W. & Pearson, C. M. *Brain* **92**, 9-24 (1969).
4. Dubowitz, V. *Muscle Disorders in Childhood*, 146-178 (Saunders, London and Philadelphia, 1978).
5. Pearn, J. H., Carter, C. O. & Wilson, J. *Brain* **96**, 463-470 (1973).
6. Pearn, J. H., Hudson, P. & Walton, J. N. *Brain* **101**, 591-606 (1978).
7. Kennedy, W. B., Alter, M. & Sung, J. G. *Neurol. Minn.* **18**, 670-680 (1968).
8. Dubowitz, V. *Brain* **87**, 707-718 (1964).
9. Fried, K. & Emery, A. E. H. *Clin. Genet.* **2**, 203-209 (1971).
10. Pearn, J. H., Carter, C. O. & Wilson, J. *Brain* **96**, 463-470 (1973).
11. Pearn, J. H. & Wilson, C. O. *Archs Dis. Childh.* **48**, 768-774 (1973).
12. Pearn, J. H., Gardner-Medwin, D. & Wilson, J. F. J. *Neurol. Sci.* **37**, 227-248 (1978).
13. Ott, J. *Analysis of Human Genetic Linkage* (Johns Hopkins University Press, Baltimore, 1985).
14. Lander, E. S. & Botstein, D. *Genetics* **121**, 185-199 (1989).
15. Love, D. R. *et al. Br. med. Bull.* **45**, 659-680 (1989).
16. Keats, B., Ott, C. & Conneally, M. *Cytogenet. Cell Genet.* **51**, 459-502 (1989).
17. Guiffra, L. A. *et al. Cytogenet. Cell Genet.* **49**, 313-314 (1988).
18. Johnson, W. G. *et al. Ann. Neurol.* **11**, 11-16 (1981).
19. Cashman, N. R. *et al. Ann. Neurol.* **19**, 568-572 (1986).
20. Lathrop, G.M., Lalouel, J. M., Julier, C. & Ott, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3443-3446 (1984).
21. Gilliam, T. C. *et al. Genomics* **5**, 940-944 (1989).
22. Leppert, M. *et al. Science* **238**, 1411-1413 (1987).

ACKNOWLEDGEMENTS. We are indebted to the SMA families whose cooperation and support made this project possible, and also to the early contributions of Dr Michael Mendelsohn. Thanks to Barbara Byth, Dr Sally Candy, and Bartolome Jaume-Roig for technical assistance, Linda Skerry for family coordination, Dr Kamma Das for tissue culture, and Dr Lewis P. Rowland for helpful discussions. This work was supported by the Muscular Dystrophy Association of America, the Muscular Dystrophy Group of Great Britain and Northern Ireland, the MRC of Great Britain, and the W. M. Keck Foundation.

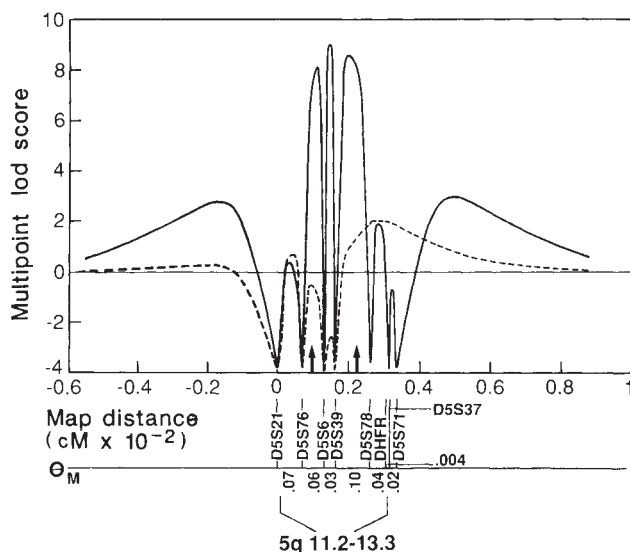


FIG. 1 Multipoint linkage analysis of the SMA disease locus with eight DNA markers spanning ~30 cM, including 5q11.2-5q13.3 (refs 16, 21). Analysis of seven chronic families (solid line) and six acute families (dotted line). Three chronic families each consists of four affected children and 8-12 unaffected sibs. Four chronic families each have three affected children and 0-4 unaffected sibs. The acute families, collected over a 3-year period, include one family with three affected children (trizygotic triplets), four families with two affected children, and one family with one affected and two unaffected sibs. Recombination fractions (θ_M) between DNA markers were calculated from published map distances¹⁶. Marker loci D5S6, D5S39, D5S78 and DHFR map to 5q11.2-13.3 (ref. 21). For the female-to-male distance ratio we used the published value of 1.6 as being appropriate for this area of the genome²². Multipoint lod scores were obtained by five-point analysis in all families, except one for which, for reasons of computational efficiency, three-point lod scores had to be calculated. The computer program used was LINKMAP of the LINKAGE package²⁰. The confidence interval for chronic families (defined as points on the map with lod scores $\geq Z_{\max}^{-1}$ where $Z_{\max}$ is the value of $Z(\theta)$ at the maximum likelihood estimate of θ) spans an 11-cM region marked by arrows at map positions 0.11 and 0.22.

Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene

J. J. Mullins, J. Peters & D. Ganten

German Institute for High Blood Pressure Research and Department of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, D-6900 Heidelberg, FRG

PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized¹⁻⁸, including those of the renin-angiotensin system, which plays a central part in blood pressure control⁹⁻¹⁰. Here we describe the introduction of the mouse *Ren-2* renin gene^{3,11-13} into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

We chose the mouse *Ren-2* renin gene for introduction into the rat germline because it had already been characterized in transgenic mice and because we expected it to be highly

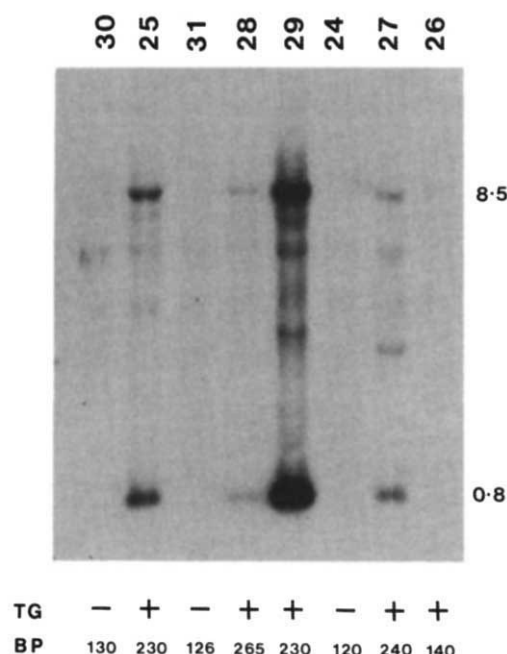


FIG. 1 Southern blot identifying animals carrying the DBA/2J *Ren-2* gene. The identification numbers of potential founder animals are shown above the corresponding lane and the positions of the *Ren-2*-specific 8.5-kb and 0.8-kb restriction fragments are indicated to the right. Transgenic (TG) positive and negative animals are indicated by symbols under the corresponding lane, together with the systolic blood pressure (BP, in mm Hg) of each animal at the age of 10 weeks.

METHODS. DNA preparation: DNA was prepared from tail biopsies and digested with *PvuII*. After electrophoresis on a 0.8% agarose gel, samples were Southern-blotted and hybridized with a ^{32}P -labelled dCTP 300-bp probe derived from the renin complementary DNA clone pDD1D2¹⁷ and labelled by random priming¹⁸. Preparation of transgenic animals: DNA was prepared for microinjection by digestion of the cosmid clone cosDBA-1 (ref. 17) with *XhoI*, and subsequent isolation of the 24-kb *XhoI* fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol-precipitation before being centrifuged on a CsCl gradient¹⁹. DNA was diluted to a final concentration of $1 \mu\text{g ml}^{-1}$ in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at -20°C before use. Fertilized eggs were derived from a cross between Sprague-Dawley female and WKY male rats after superovulation of immature females (at 4 weeks old) according to the procedure of Armstrong *et al.*²⁰. Eggs were cultured, microinjected, and re-implanted as described for the mouse¹⁹. Rats were all bred in our own facilities.

expressed in certain tissues¹⁴; also, injection of purified mouse submandibular gland (SMG) renin (encoded by *Ren-2*) into rats leads to a significant and sustained increase in blood pressure¹⁵. Fertilized rat eggs were microinjected with a linear DNA fragment containing the entire DBA/2J *Ren-2* gene, including 5.3 and 9.5 kilobases (kb) of 5' and 3' flanking sequence, respectively¹⁴. From 37 eggs implanted, there were eight progeny, of which five carried the transgene (Fig. 1). Four of the founders were bred successfully and three of them (TGRmRen2, numbers 25, 26 and 27) transmitted the transgene to their progeny. At ten weeks of age and before breeding, the blood pressure of the founder animals was measured. For four of the transgenic animals it was in the range 230–265 mm Hg, but was 120–130 mm Hg in the transgene-negative litter-mates (Fig. 1). Breeding of TGRmRen2 female 26, who was not hypertensive, revealed her to be mosaic for a transgene insertion site, the inheritance of which segregated with hypertension in the blood pressure range indicated (data not shown). The phenotype is therefore independent of the transgene insertion site and is not due to a fortuitous mutation associated with the integration event.

Analysis of the transgenic line established from TGRmRen2 male 27 revealed that, without exception, progeny inheriting the transgene also had the hypertensive phenotype. Both male and female animals of this line developed hypertension rapidly, beginning at four weeks of age and reaching a maximum by nine weeks (Fig. 2a). Pharmacological intervention to reduce

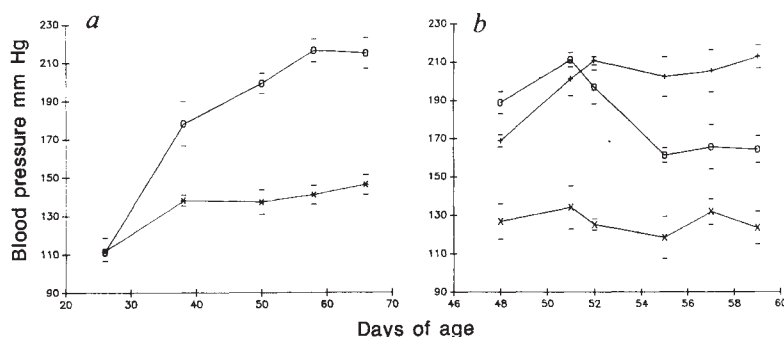
blood pressure took the form of treating the animals with 10 mg kg^{-1} per day of the converting enzyme inhibitor, captopril; this inhibits the conversion of angiotensin I to angiotensin II. This low dose, given daily in the drinking water, was sufficient to reduce the blood pressure of the hypertensive transgenic rats reproducibly by 40–60 mm Hg (Fig. 2b), indicating that the hypertension is largely dependent on the conversion of angiotensin I to angiotensin II.

Northern blot analysis showed that the concentration of renin transcripts was high in the adrenal glands of the transgenic animals (Fig. 3a). In addition, renin transcripts were detectable in testis, coagulation gland, thymus and small intestine in transgene-positive animals, but not in control transgene-negative littermates (data not shown). These additional sites represent tissues in which renin is naturally expressed in the mouse. Renin messenger RNA was not observed in the SMG, a result that could reflect the absence of essential *trans*-acting factors in this tissue as the endogenous rat renin gene is not expressed in the SMG (ref. 16). An RNase protection assay using a *Ren-2*-specific probe confirmed that the renin transcripts in the adrenal gland were exclusively of *Ren-2* origin and that *Ren-2* transcripts were present in the kidneys of transgene-positive animals (Fig. 3b).

No evidence was found for altered plasma angiotensinogen levels, but plasma renin activity and angiotensin I were significantly lower in transgenic animals than in the controls (Fig. 4b–e). The amount of angiotensin II was also less than in the

FIG. 2 a, Development of blood pressure with age. Each point represents the mean of 7 (transgenic, circles) or 5 (control, crosses) animals and standard errors are indicated above and below each data point. b, Effect of converting enzyme inhibitor (CEI) on blood pressure. Each point represents the mean of 3 animals and standard errors are indicated above and below each data point. +, TGRmRen2 L27 rats having no treatment; ○, TGRmRen2 L27 rats receiving CEI; ×, control rats receiving CEI.

METHODS. Blood pressure was determined by tail plethysmography under light ether anaesthesia as described²¹. Animals under converting enzyme inhibitor treatment were given captopril (10 mg kg^{-1} per day) in their drinking water. Captopril treatment started at day 51.



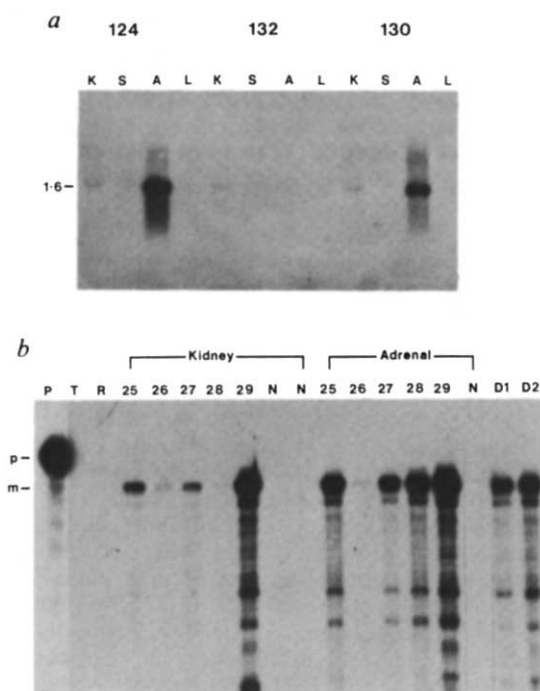


FIG. 3 Northern blot and RNase protection assay. *a*, Northern blot of RNA isolated from the kidney (K); SMG (S); adrenal gland (A); and liver (L) of transgene-positive (124 and 130) and transgene-negative (132) male rats. The size of the hybridizing RNA is indicated in kb. With the exception of the adrenal gland (5 μ g), 40 μ g total RNA was used for each sample. *b*, RNase protection of kidney and adrenal gland RNA from transgenic rats (numbers 25–29) and control littermates (N). The following controls are included: P, undigested probe; T, transfer RNA (9 μ g); R, rat kidney RNA (20 μ g); D1 and D2, mouse (DBA/2J) kidney RNA (20 μ g and 40 μ g, respectively). The positions of the undigested 244-nucleotide probe (p) and the 224-nucleotide mouse-specific protected fragment (m) are indicated.

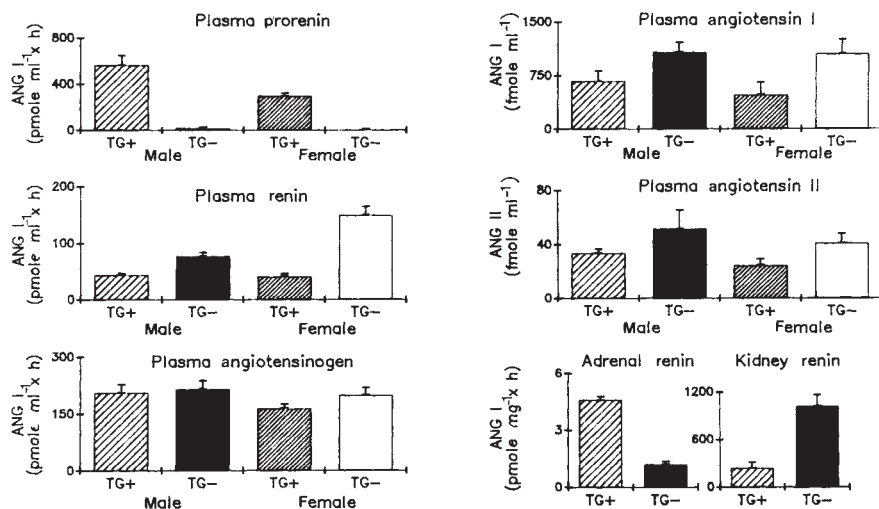
METHODS. Preparation of RNA: Total RNA was isolated from mature rats as previously described³ or by homogenization in guanidine isothiocyanate.²² Northern blot analysis: Northern blots were prepared and hybridized as previously described²³ with a ³²P-labelled renin cDNA probe (pDD1D2) by random priming¹⁸ and washed with 0.1 $\times$ SSC, 0.1% SDS at 65 °C. RNase protection assay: ³²P-labelled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM (ref. 15) using SP6 RNA polymerase. This transcript comprised 224 nucleotides of *Ren-2* antisense RNA and 20 nucleotides of vector-encoded sequence. Samples were dissolved in 30 μ l 80% formamide, containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA and 200,000 c.p.m. of the gel-purified transcript, denatured at 100 °C for 1 min and incubated at 45 °C for 20 h. RNase digestion was performed in 300 μ l buffer containing 40 μ g ml⁻¹ RNase A (Sigma) and 2 μ g ml⁻¹ RNase T1 (Calbiochem) for 45 min at 37 °C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels.

controls but the difference was not statistically significant. Determination of prorenin showed it to be raised in the plasma of transgenic animals (Fig. 4a), but the functional significance of this finding is unclear. Adrenal glands of the transgenic animals contained significantly increased renin concentrations (Fig. 4f). No evidence was found for the storage of renin in this tissue, so the large difference between renin mRNA levels and enzyme activity may reflect a constitutive secretion of *Ren-2*-derived renin from the adrenal glands. By contrast, kidney tissue from transgenic animals contained only 20–25% of the renin activity of the controls, which is consistent with immunocytochemical and ultrastructural data showing a reduction in renin storage granules in the juxtaglomerular apparatus (S. Bachmann *et al.*, manuscript in preparation) and suggests that renin expression is subject to translational or post-translational control. Preliminary studies on isolated kidney show that renin secretion is reduced and that there are no other abnormalities of renal function (K. Munter, personal communication).

Although we have defined a genetic basis for this transgenic hypertensive rat model, the mechanism responsible for elevating blood pressure remains to be established. The hypertension is clearly not due to overexpression of renin in the kidney, and the suppression of active renin in the kidney and in the plasma is probably a result of an already elevated blood pressure in young animals, pressure-mediated renin suppression being a well known phenomenon. The increased plasma prorenin probably originates, at least in part, from the adrenal gland, but the ovary, vascular tissue and other sources of prorenin should also be considered. Any role of prorenin in hypertension still awaits investigation, but in this respect it is interesting that prorenin is raised and still persists after nephrectomy in hypertensive patients, confirming that its origin is extra-renal. At this stage, the most likely explanation for the high blood pressure in TGRmRen2 rats is a stimulated renin-angiotensin system in the adrenal gland, with the consequent overproduction of steroid hormones. This is in keeping with our preliminary data on

FIG. 4 Determination of plasma and tissue renin-angiotensin system components. Values represent the mean and standard error of 7 animals for each determination, with the exception of the kidney and adrenal gland renin values (3 animals). Statistical analysis by ANOVA showed the following significance values: prorenin, $P < 0.05$ between the transgenic animals and the corresponding controls; renin, $P < 0.005$ between the transgenic animals and the corresponding controls; angiotensin I, $P < 0.05$ between the transgenic animals and the corresponding controls; tissue renin, $P < 0.01$ for the adrenal gland and $P < 0.005$ for the kidney.

METHODS. Concentrations of angiotensinogen, angiotensin I, angiotensin II and renin were determined as described^{24–25}. Prorenin levels were calculated by subtraction of renin activity from total plasma renin activity determined after trypsin activation²⁶.



elevated urinary aldosterone excretion in male TGRmRen2 rats (15.4 ± 2.26 ng per 24 h) compared with controls (8.97 ± 1.06 ng per 24 h). These animals will enable us to study normal or low plasma renin hypertension and have shown us that renin can participate in the genesis of hypertension in a more subtle way than previously supposed. The construction of transgenic rats will therefore provide new opportunities for research into cardiovascular mechanisms. □

Received 14 November 1989; accepted 9 February 1990.

1. Miyazaki, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 5999–6003 (1984).
2. Burnham, C. E., Hawelu-Johnson, C. L., Frank, B. M. & Lynch, K. R. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5605–5609 (1987).
3. Mullins, J. J. *et al. EMBO J.* **1**, 1461–1466 (1982).
4. Gaillard, I., Clauser, E. & Corvol, P. *DNA* **8**, 87–89 (1989).
5. Kageyama, R., Ohkubo, H. & Nakanishi, S. *Biochemistry* **23**, 3603–3609 (1984).
6. Seidman, C. E., Bloch, K. D., Klein, K. A., Smith, J. A. & Seidman, J. G. *Science* **226**, 1206–1209 (1984).
7. Schmale, H., Iweli, R., Breindl, M., Darmer, D. & Richter, D. *EMBO J.* **3**, 3289–3293 (1984).
8. Burt, D. W. *et al. in Aspartic Proteinases and their Inhibitors* (ed. Kostka, V.) 355–377 (de Gruyter, Berlin, 1985).
9. Peach, M. *Physiol. Rev.* **57**, 313–370 (1977).
10. Campbell, D. G. *J. clin. Invest.* **79**, 1–6 (1987).
11. Panthier, J. J., Holm, T. & Rougeon, F. *EMBO J.* **1**, 1417–1421 (1982).
12. Piccini, N., Knopf, J. L. & Gross, K. W. *Cell* **30**, 205–213 (1982).
13. Field, L. J. & Gross, K. W. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6196–6200 (1985).
14. Mullins, J. J., Sigmund, C. D., Kane-Haas, C. & Gross, K. W. *EMBO J.* **8**, 4065–4072 (1989).
15. Onoyama, K., Omae, T. & Inagami, T. *Jap. Heart J.* **19**, 522–530 (1978).
16. Ekker, M., Tronik, D. & Rougeon, F. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5155–5158 (1989).
17. Field, L. J. *et al. Molec. cell. Biol.* **4**, 2321–2331 (1984).
18. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6–13 (1983).
19. Hogan, B., Constantini, F. & Lacy, E. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).
20. Armstrong, D. T. & Opavsky, M. A. *Biol. Reprod.* **39**, 511–518 (1988).
21. Rascher, W., Clough, D. & Ganten, D. in *Hypertensive Mechanisms* 775–801 (Schattauer, Stuttgart, 1982).
22. Chirgwin, J. A., Przbyla, A., McDonald, R. & Rutter, W. J. *Biochemistry* **18**, 5294–5299 (1979).
23. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).
24. Schelling, P., Ganten, U., Sponer, G., Unger, T. & Ganten, D. *Neuroendocrinology* **31**, 297–308 (1980).
25. Hermann, K., Ganten, D., Unger, T., Bayer, C. & Lang, R. E. *Clin. Chem.* **34**, 1046–1051 (1988).
26. Glorioso, N. *et al. Clin. Sci.* **64**, 137–140 (1983).

ACKNOWLEDGEMENTS. We thank Dr K. W. Gross for pCOS-1 and pSLM, Dr D. Armstrong for advice and communication of unpublished results, Frank Zimmerman and Gesa Wernicke for technical assistance, Dr L. J. Mullins for critically reading the manuscript, and Drs U. Ganten, Yi Zhao and M. Lee for their contributions. This work was supported in part by the Deutsche Forschungsgemeinschaft, Squibb/von Heyden Co., Munich and The Commission of the European Communities, Concerted Action Program TRANSGENEUR.

Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses

H. R. Brenner*, V. Witzemann†
& B. Sakmann†

*Physiologisches Institut, Universität Basel, Vesalgasse 1, 4051 Basel, Switzerland

†Max-Planck-Institut für Medizinische Forschung, Abteilung Zellphysiologie, 6900 Heidelberg, Jahnstrasse 29, FRG

IN mammalian muscle, the subunit composition of the nicotinic acetylcholine receptor (AChR) and the distribution of AChRs along the fibre are developmentally regulated. In fetal muscle, AChRs are distributed over the entire fibre length whereas in adult fibres they are concentrated at the end-plate¹. We have used *in situ* hybridization techniques to measure the development of the synaptic localization of the messenger RNAs (mRNAs) encoding the α -subunit and the ϵ -subunit of the rat muscle AChR. The α -subunit is present in both fetal and adult muscle, whereas the ϵ -subunit appears postnatally and specifies the mature AChR subtype^{2–4}. The synaptic localization of α -subunit mRNA in adult fibres may arise from the selective down-regulation of constitutively expressed mRNA from extrasynaptic fibre segments. In contrast,

ϵ -subunit mRNA appears locally at the site of neuromuscular contact and its accumulation at the end-plate is not dependent on the continued presence of the nerve terminal very early during synapse formation. This suggests that ϵ -subunit mRNA expression is induced locally via a signal which is restricted to the end-plate region and is dependent on the presence of the nerve only during a short period of early neuromuscular contact. Evidently, several mechanisms operate to confine AChR mRNAs to the adult end-plate region, and the levels of α -subunit and ϵ -subunit mRNAs depend on these mechanisms to differing degrees.

Hybridization of longitudinal sections of adult rat soleus muscle with ϵ - and α -subunit-specific antisense complementary RNA (cRNA) probes revealed strong hybridization signals at sites that had been previously identified as end-plates by staining for acetylcholinesterase (AChE). Figure 1a shows the end-plate region of a muscle stained for AChE. Subsequent hybridization with the ϵ -subunit-specific antisense probe showed a strong signal at the site where the AChE stain had been (Fig. 1b). After a brief exposure, groups of grains could be resolved above individual synaptic nuclei (Fig. 1c); no hybridization was observed outside end-plate regions. When sections were incubated with ϵ -subunit-specific sense probes, no hybridization could be detected (data not shown). These observations suggest that autoradiographic grain clusters reflect locally increased ϵ -subunit mRNA levels below the end-plate membranes. Similar results were obtained after hybridization with α -subunit-specific antisense (Fig. 1d, e) and sense probes and confirm the synaptic localization of α -subunit mRNA in rat muscle, as observed previously using northern blot analysis⁵. In some fibres, a small signal was observed above nuclei in the perijunctional region of the muscle fibres (Fig. 1e).

Previous northern blot analysis of AChR-specific mRNAs in neonatal rat muscle indicated that ϵ -subunit mRNA is barely detectable at birth but that levels increase rapidly during the first 2 weeks of postnatal development⁴. To determine whether this increase in ϵ -subunit mRNA is restricted to the end-plate region and therefore would be induced focally by the nerve, or whether the increase is more general, involving the entire fibre, we hybridized triceps muscle from rats of different postnatal ages with an ϵ -subunit mRNA-specific cRNA probe. Figure 2a shows the localization of AChE and autoradiographs of longitudinally sectioned muscle (b–d). At postnatal day 1, no hybridization signal could be detected either synaptically or extrasynaptically (Fig. 2b). In dark-field microscopy, some of the synaptic sites revealed a weak accumulation of grains (data not shown). However, on postnatal days 5, 9 (data not shown) and 12, an increasingly stronger signal was seen (Fig. 2c) that always coincided with the AChE-stained synaptic sites. Thus, the postnatal appearance of ϵ -subunit mRNA is restricted to the end-plate region from the earliest stages of synapse development and therefore must be induced by the nerve-muscle contact. As in adult muscle, hybridization signals in postnatal day-12 muscles were clearly associated with individual nuclei, as shown in Fig. 3. However, given the high density of nuclei from various cell types, unequivocal attribution to subneural nuclei was not always possible.

In contrast, total α -subunit mRNA remained at a plateau level during the first 12 postnatal days⁴. During this period, the α -subunit mRNA was detected throughout the fibre, in both the synaptic and extrasynaptic fibre segments (Fig. 2f, g). Although there were more grains at the synaptic sites, they were more widely distributed than those obtained upon hybridization with the ϵ -subunit mRNA specific probe. Moreover, the hybridization signal was also observed outside the myofibre bundles above unfused cells.

The level of total muscle ϵ -subunit mRNA increases almost normally in neonatal muscle denervated shortly after birth⁴, indicating that only the brief, prenatal nerve-muscle contact is necessary to induce ϵ -subunit mRNA synthesis. We have investigated whether the ϵ -subunit mRNA still appears focally at the

FIG. 1 Localization of ϵ -subunit and α -subunit mRNA at neuromuscular synapses of longitudinal sections of adult rat soleus muscle. *a, d*, Staining of end-plate regions for AChE using Nomarski optics. *b*, Same region as in *a*, shown after hybridization with antisense ϵ -subunit-specific cRNA probe and staining of nuclei with haematoxylin. Comparison of 203 AChE-positive sites and 204 ϵ -subunit mRNA-positive sites showed a correlation of about 92% between the two. Grain accumulations without concomitant AChE stain deposits were usually observed in the vicinity of clusters of AChE-stain-identified endplates. These accumulations probably represent end-plates whose stain deposits had been removed by sectioning. *c*, Same synapse shown at a higher magnification to illustrate perinuclear accumulation of grains. *e*, Same region as in (*d*), is shown after hybridization with antisense α -subunit-specific cRNA probe and staining with haematoxylin. Note the grain accumulations above perijunctional nuclei marked by arrows. Scale bars, 70 μm (*a, b, d, e*) and 175 μm (*c*).

METHODS. We used soleus muscles of adult rats of 200–300 g. The muscles were fixed and stained for acetylcholinesterase (AChE) as previously described¹⁷. Sections of 8–10 μm were mounted on aminoalkylsilane-treated glass slides¹⁸. After rehydration, the sections were scanned for end-plate AChE stain deposits and their locations with respect to the scales of the microscope stage were recorded. Sections were then pretreated and hybridized, essentially as described previously^{7,8}. They were viewed on a Zeiss Standard microscope equipped with dark-field illumination and

Nomarski optics, and the locations of grain accumulations were compared with those of the AChE-positive sites. Full-length complementary DNA (cDNA) clones of the rat α - and ϵ -subunits consisting of 1844 base pairs (bp) and 1624 bp (Witzemann *et al.*, manuscript in preparation) were subcloned into the polylinker region of the transcriptional vectors pSP64 and pSP65 (Promega)¹⁹. [³⁵S]-labelled sense and antisense cRNA was prepared using SP6 polymerase (Promega) and [³⁵S]uridine 5'-triphosphate, (40 mCi ml⁻¹, Amersham), following procedures previously described⁷.

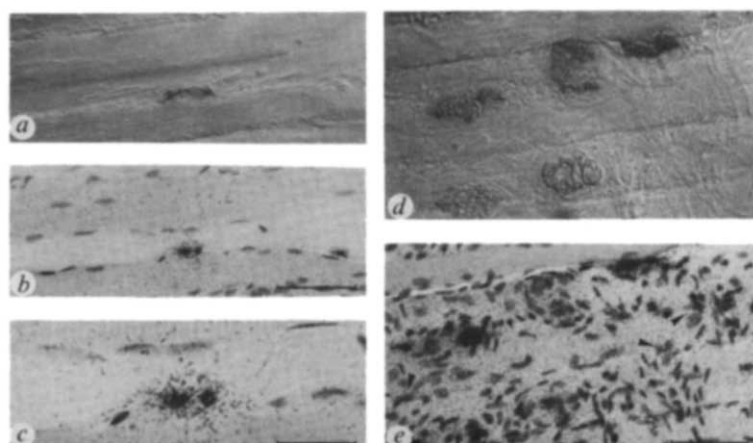
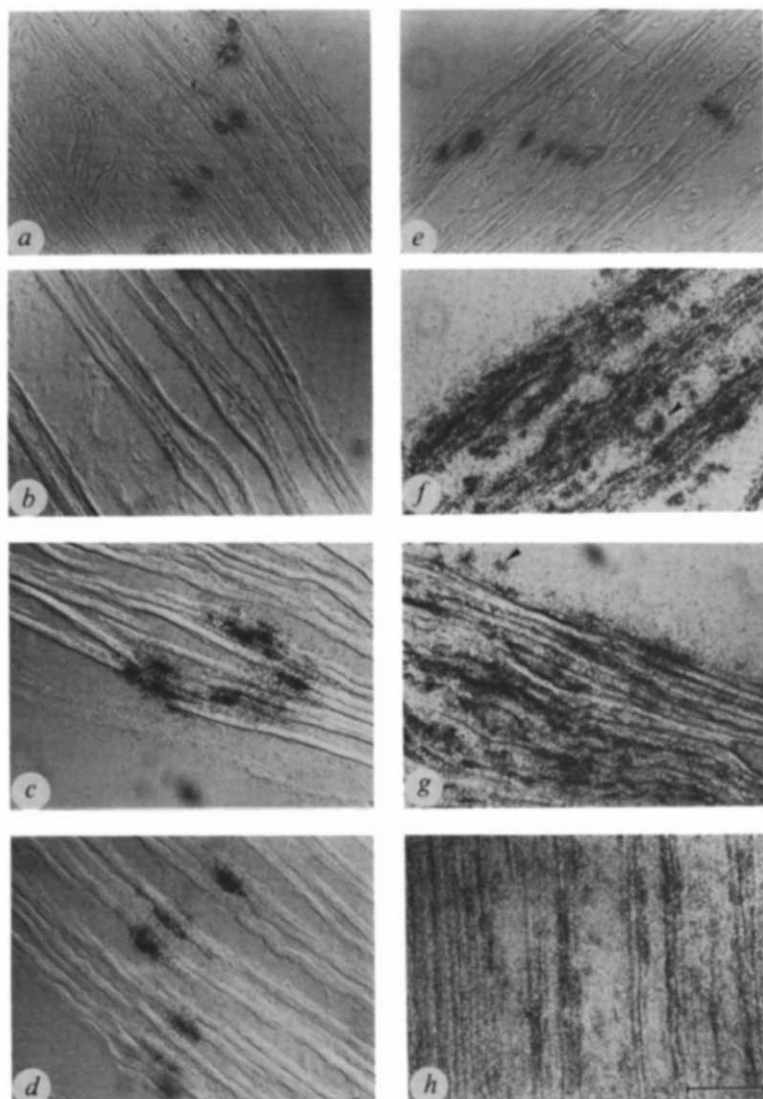


FIG. 2 The distribution of ϵ - and α -subunit mRNA in longitudinal sections of neonatal rat triceps muscle. *a*, Staining of end-plate regions for AChE at postnatal day 1. *b*, Same region as in *a*, shown after hybridization with the ϵ -subunit mRNA antisense probe; Nomarski photograph showed no increase in grain density in synaptic areas. *c*, Section from postnatal day 12 triceps muscle after hybridization with the ϵ -subunit mRNA antisense probe. *d*, Hybridization with ϵ -subunit mRNA antisense probe of postnatal day 9 triceps muscle which had been denervated at postnatal day 1. (*e–h*) Same protocol as (*a–d*) except that sections were hybridized with the α -subunit mRNA antisense probe. Note grain accumulations outside unfused cells (arrows). Hybridizations of 1- and 12-day-old muscles were carried out in the same experiment with the same batches of ϵ - and α -subunit mRNA antisense probes, respectively. Synaptic sites in (*c, d, g, h*) had been identified by AChE staining before hybridization. Scale bar, 70 μm .

METHODS. Experiments were carried out on triceps muscles of neonatal rats. Denervation of muscles was carried out under ether anaesthesia, excising a 1–5 mm piece of the sciatic nerve. In neonatal animals, surgery was performed unilaterally within 1 hour of birth. Eight days later, the denervated state of the leg was ascertained clinically by the missing toe spreading reflex and by the absence of a pain reflex upon prodding the footpads with a fine needle. The same reflexes were elicited from the unoperated contralateral side for comparison. Animals were then killed by decapitation and denervated muscles and muscles from unoperated littermates were processed for *in situ* hybridization. To examine either developmental changes with age or the effects of denervation, sections from muscles to be compared were hybridized with the same batch of probe and were processed in the same experiment.



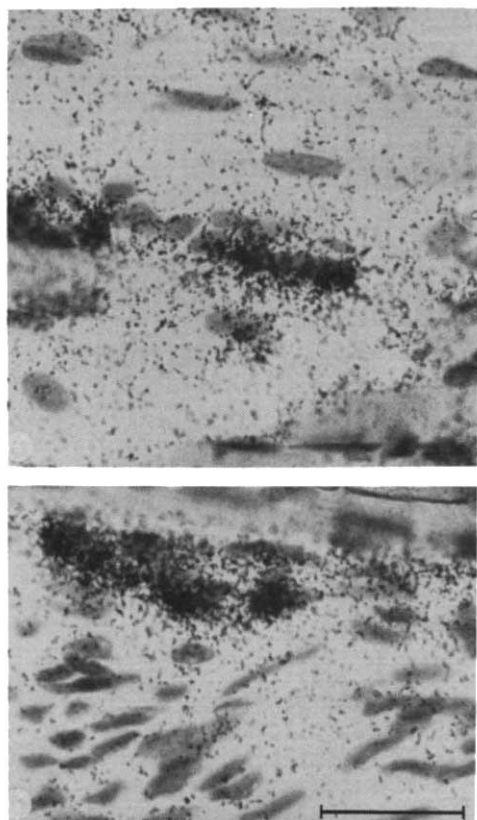


FIG. 3 Perinuclear accumulation of ϵ -subunit mRNA in neonatal triceps muscle. Examples of perinuclear accumulation of grains in postnatal day 12 triceps muscle hybridized with ϵ -subunit mRNA antisense probe. Nuclei of two different fibres are visualized by staining with haematoxylin. Scale bar, 175 μ m.

end-plate region in these muscles. Fig. 2d shows the autoradiograms of fibres from a muscle from a 9-day postnatal rat, which had been denervated within 1 day of birth. Clearly, even when the neural influence is removed while the junctional ϵ -subunit mRNA is still low or absent, the accumulation of ϵ -subunit mRNA occurs focally, at least up to postnatal day 9 and no extrajunctional increase in ϵ -subunit mRNA could be

resolved. Thus, postnatal accumulation does not require the continued presence of the nerve.

The more diffuse distribution of α -subunit mRNA did not significantly change after early denervation, in that grains remained distributed along the entire fibre length (Fig. 2h), similarly to the distribution observed in innervated fibres of the same age (Fig. 2g). This indicates that during early postnatal development innervation has little effect on the distribution of α -subunit mRNA along the fibre length. However, sections from denervated muscles showed a higher extrajunctional grain density than control muscles of the same age, often making the location of synaptic α -subunit mRNA very difficult.

As the induction of ϵ -subunit mRNA at neonatal end-plates is resistant to denervation at early stages of end-plate formation, the question arises of how stable the end-plate-specific accumulation of ϵ -subunit mRNA is in adult muscle after removal of the nerve. Figure 4(a, b) shows that after 14 days of chronic denervation, the ϵ -subunit mRNA remained largely restricted to the end-plate region, as was observed in innervated control muscle (Fig. 1b, c). In contrast, when denervated soleus fibres were hybridized with the α -subunit mRNA-specific probe (Fig. 4c), a drastic increase in α -subunit mRNA in extrasynaptic fibre segments was apparent. This was particularly obvious around the extrajunctional nuclei after 5 days of denervation (Fig. 4d).

In adult rat muscle, both α - and ϵ -subunit mRNAs are localized around end-plate nuclei, as shown previously for α -subunit mRNA in rat⁶ and chicken muscle^{7,8}. However, the mechanisms leading to this focal accumulation are different for the α - and ϵ -subunit mRNAs. Two superimposed mechanisms could regulate the expression of the α -subunit mRNA: one inhibits expression, depends on muscle activity and operates on the entire muscle fibre⁸⁻¹⁰; the other promotes expression, is confined to the end-plate and may be similar to the regulation of ϵ -subunit mRNA described here. Local up-regulation of α -subunit mRNA at the synapse by neurotrophic factors¹¹⁻¹³ remains to be demonstrated. In contrast, ϵ -subunit mRNA is expressed primarily at the end-plate from the time it first appears a few days after birth. The synaptic localization of ϵ -subunit mRNA is caused by a brief contact of nerve and muscle during the first 3-4 days of synapse formation, assuming that the muscles of the hindleg become innervated at the 17th day of gestation^{14,15}. After this initial imprinting, the accumulation of ϵ -subunit mRNA does not depend on the continued presence of the nerve terminal.

In both neonatal and adult muscle hybridized with the ϵ -subunit mRNA-specific probe, autoradiographic grains were

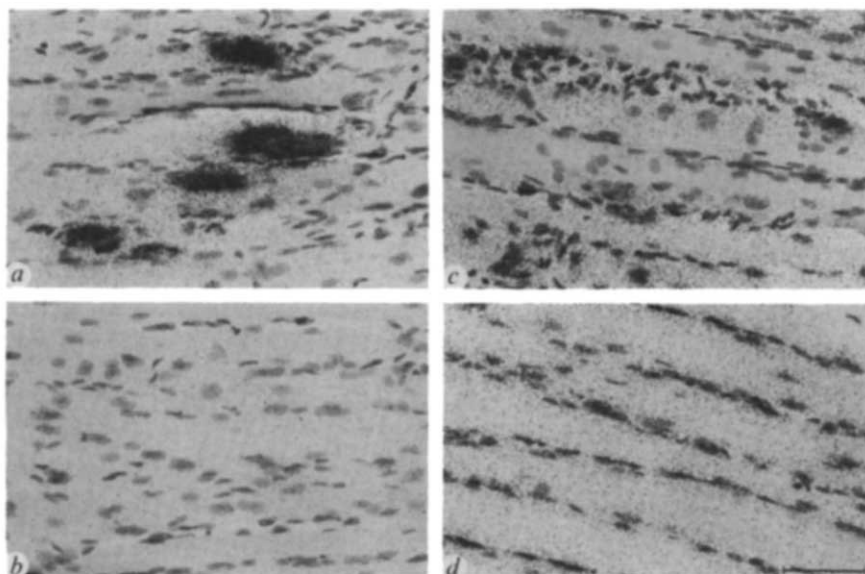


FIG. 4 Effect of denervation on distribution of AChR mRNAs in adult rat soleus muscle. a, ϵ -subunit mRNA in synaptic region of muscle after 14 days of chronic denervation. b, Extrasynaptic area from same section as in (a). Note that expression of ϵ -subunit mRNA remains localized at the synapse. c, α -subunit mRNA in synaptic region of muscle after 5 days of denervation. d, Extrasynaptic region of the same section as in (c). Note the significant increase of α -subunit mRNA accumulations around extrasynaptic nuclei. Scale bar, 70 μ m. Adult animals were denervated unilaterally (see Fig. 2) and killed with ether after 5 or 14 days.

associated with single nuclei. This suggests that the synaptic localization of ϵ -subunit mRNA arises from its accumulation around specific end-plate nuclei, rather than from many weakly active nuclei accumulating at the end-plate as it develops¹⁶. One remaining question is whether the accumulation of perinuclear ϵ -subunit mRNA is due to increased transcriptional activity by the end-plate nuclei or to its local metabolic stabilization. □

Received 10 October 1989; accepted 8 January 1990.

- Schuetze, S. M. & Role, L. W. *Ann. Rev. Neurosci.* **10**, 403–457 (1987).
- Mishina, M. *et al. Nature* **321**, 406–411 (1986).
- Gu, Y. & Hall, Z. W. *Neuron* **1**, 117–125 (1988).
- Witzemann, V., Barg, B., Criado, M., Stein, E. & Sakmann, B. *FEBS Lett.* **242**, 419–424 (1989).
- Mertle, J. P. & Sanes, J. R. *Nature* **308**, 653–655 (1985).
- Goldman, D. & Staple, J. *Neuron* **3**, 213–228 (1989).
- Fontaine, B., Sassoon, D., Buckingham, M. & Changeux, J. P. *EMBO J.* **7**, 603–609 (1988).
- Fontaine, B. & Changeux, J. P. *J. cell Biol.* **108**, 1025–1037 (1989).
- Klarsfeld, A. & Changeux, J. P. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4558–4562 (1985).
- Goldman, D., Brenner, H. R. & Heinemann, S. *Neuron* **1**, 329–333 (1988).
- Fontaine, B., Klarsfeld, A. & Changeux, J. P. *J. cell Biol.* **105**, 1337–1342 (1987).
- Harris, D. A., Falls, D. L., Dill-Devor, R. M. & Fischbach, G. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1983–1987 (1988).
- Harris, D. A., Falls, D. L. & Fischbach, G. D. *Nature* **337**, 173–176 (1989).
- Angulo-Y-Gonzales, A. W. *J. comp. Neurol.* **55**, 395–442 (1932).
- Bevan, S. & Steinbach, J. H. *J. Physiol., Lond.* **267**, 195–213 (1977).
- Phillips, W. D. & Bennett, M. R. *J. Neurocytol.* **18**, 241–255 (1989).
- Koelle, G. B. & Friedenwald, J. S. *Proc. Soc. Biol. Med.* **70**, 617–622 (1949).
- Rentrop, M., Knapp, B. H., Winter, H. & Schweizer, J. *Histochem. J.* **18**, 271–276 (1986).
- Melton, D. A. *et al. Nucleic Acids. Res.* **12**, 7035–7056 (1984).

ACKNOWLEDGEMENTS. This work was supported by grants from the Schweiz. Nationalfonds and the Sandoz-Stiftung für Förderung der Medizinisch-Biologischen Wissenschaften, Basel. We thank Drs B. Fontaine and J. P. Changeux for advice with *in situ* hybridization and E. Stein for technical assistance.

Sexual development in the pea is presaged by altered expression of arabinogalactan protein

Roger I. Pennell* & Keith Roberts

Department of Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

FLOWERS contain the reproductive cells of angiosperms. Floral meristems develop apically or subapically from vegetative axes, and the flower itself is often regarded as homologous with a compressed and determinate shoot¹. Although in many extant angiosperms the fertile floral organs (stamens and carpels) resemble leaves in both development and morphology², the structures that give rise to the germ cells inside them (the anther and ovule) have no counterpart in any vegetative organ. The reproductive cells of angiosperms therefore develop after a period of exclusively vegetative growth and, in contrast to those of animals, have no direct antecedents in the embryo³. So far, the molecular mechanisms that set the reproductive cells apart from those of the other components of the flower or from the vegetative organs are unknown. Here we describe an early and specific developmental switch in stamen and carpel primordia of pea (*Pisum sativum* L.) which alters expression of a plasma membrane arabinogalactan protein⁴ epitope in the progenitors of the germ cells, and which subsequently affects the gametes, zygote and globular-stage embryo.

The monoclonal antibody MAC 207 recognizes an L-arabinose-containing epitope common to a family of AGPs that are only found in the plasma membranes of flowering plants⁵. The MAC 207 epitope is present in all cells of vegetative

meristems, primordia and organs (stem, leaf and root) of *Pisum* (Fig. 1a), and throughout undeveloped flower buds (Fig. 1b), but serial examination of 25 differentiating flower buds and flowers, providing more than 1,000 frozen sections of reproductive organs, showed that specific cells of developing stamens and carpels do not contain the epitope.

In the stamen, these appeared as four apical clusters when the primordium was ~450 μ m in length and already separated from the future petal, but before longitudinal differentiation had occurred (Fig. 2a). Each cluster subsequently developed into a single pollen sac containing a layer of tapetal cells and a mass of sporogenous tissue. The outer boundary of each pollen sac was clearly marked by a MAC 207-reactive epidermis and two-layered anther wall (Fig. 2b). When meiosis and the final haploid mitosis in the male gametophyte were completed, the epitope was re-expressed in the plasma membrane of the vegetative cell of the pollen grain. The epitope did not reappear in the generative cell or the sperm.

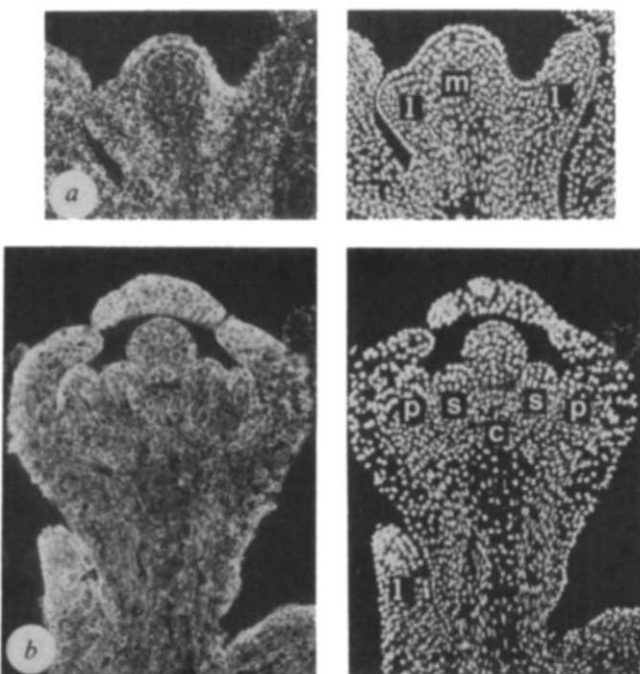


FIG. 1 Median longitudinal sections of embryonic (vegetative) apex (a) and undifferentiated flower bud (b) labelled with MAC 207 (left panels) and 4',6'-diamidino-2-phenylindole (DAPI; right panel). Cell-surface immunofluorescence is conspicuous in every cell profile, the slightly weaker staining in the sub-apical zone being due to cell expansion. Abbreviations: c, carpel primordium; l, leaf primordium; p, petal primordium; s, stamen primordium; m, shoot apical meristem. Magnification, $\times 95$.

METHODS. Vegetative tissues, developing flowers and fruits were collected from mature *Pisum sativum* L. plants (Jl 430 cv. 'Greenshaft') grown in natural light in diurnal temperature range 10–15 °C. Entire organs were fixed whole or dissected in 4% freshly depolymerized paraformaldehyde in PEM buffer (100 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid) pH 7.0, 1 mM EDTA, 1 mM magnesium sulphate) for at least 2 h at 25 °C. The tissues were washed in PEM, frozen at –10 °C in the cryoprotectant OCT Compound (Miles), and sectioned with a Bright 5030 retracting cryomicrotome at a thickness of 10 μ m. For immunofluorescence, sections were blocked with PBS pH 7.4, containing 2% ovalbumin (Sigma) for 10 min, and reacted with a 10-fold dilution (of hybridoma culture supernatant) of the anti-AGP monoclonal antibody MAC 207⁵ in the same buffer for 4 h at 25 °C. Antibody binding was identified with a rabbit anti-rat IgG H+L antiserum conjugated to fluorescein isothiocyanate (FITC; Sigma), diluted 50 times in the ovalbumin solution. Nuclei were stained by brief immersion in DAPI (1 mg l⁻¹), and fluorescence preserved by mounting in Citifluor (Citifluor, London). Primary antibody was omitted from control preparations. The microscope was a Zeiss Photomicroscope III.

* Present address: Department of Molecular Biology, Wageningen Agricultural University, POB 8091, 6700 EP Wageningen, The Netherlands.

In the carpel, the unreactive cells formed a single aggregate close to the centre of each ovule when the ovule diameter was greater than $\sim 350 \mu\text{m}$ (Fig. 3a and b). These cells ultimately developed into the embryo sac and a two-layered tissue surrounding it which was identified as the nucellus (Fig. 3a). The nucellus was enclosed in turn by a pair of MAC 207-reactive integuments (Fig. 3a). The gametophytic tissues in the ovule did not re-express the MAC 207 epitope at any stage of development. The tissues affected by this developmental switch are depicted in Fig. 4. Because the change in expression in the developing stamens coincided roughly with the appearance of ovule primordia in the carpel, MAC 207-unreactive cells were established in the future anther several days before the future nucellus could be detected in the ovule.

After fertilization, the zygote, globular-stage embryo and endosperm were entirely unreactive (Fig. 5), but the epitope reappeared throughout the embryo as it differentiated into a heart-stage structure. The epitope was first visible on about day 10 in the embryonic axis, and by day 15 in the cotyledons. Mature torpedo-stage embryos showed ubiquitous presence of the epitope.

Although the cells that do not express the MAC 207 epitope are not confined to gametophytic tissues, they are all involved with sexual reproduction. The tissues in the stamen that do not express the MAC 207 epitope form a microsporangium, whereas those of the carpel form a megasporangium². Both unreactive sporophytic structures are therefore morphologically equivalent, and to our knowledge the alteration of the cell-surface epitope expression described in this report is the first evidence of a biochemical change that specifically defines both reproductive

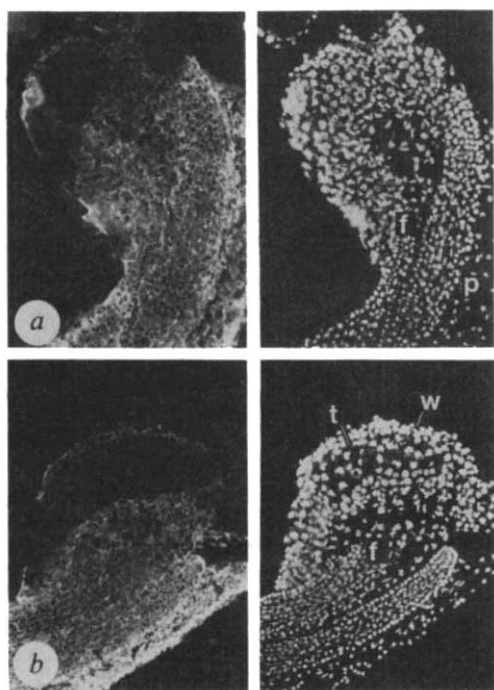


FIG. 2 Longitudinal sections of developing stamens, prepared and labelled as described in Fig. 1 with MAC 207 (left panels) and DAPI (right panels). *a*, Partially differentiated stamen primordium fixed 6–7 days before anthesis. The three unreactive cell clusters at the distal end of the organ do not correlate with recognisable tissues. Immunofluorescence comes principally from the future stamen filament (f). Petal primordium, (p). *b*, Expression of the MAC 207 epitope in a single meiosis-stage anther, fixed 2 days before anthesis. Neither the sporangium wall (w), the tapetum (t), nor the meiocytes in the anther locule contain the epitope. Fluorescing filament, f. Magnification, $\times 95$.

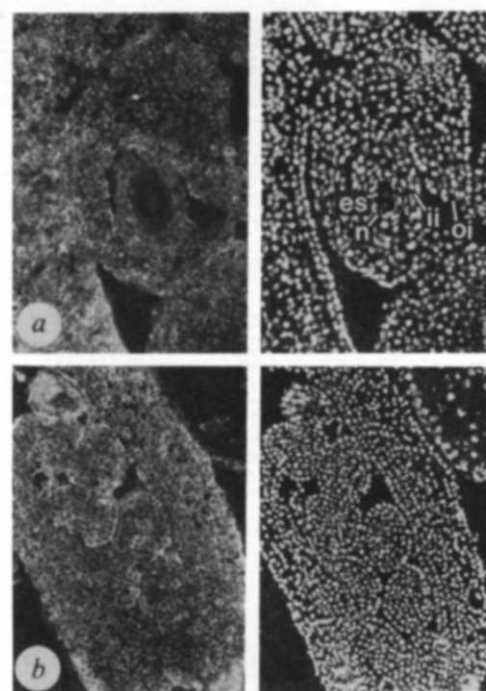


FIG. 3 Median longitudinal section of developing carpels, fixed and labelled as described in Fig. 1 with MAC 207 (left panels) and DAPI (right panels). *a*, Mature ovule. Cells that do not express the MAC 207 epitope are those of the embryo sac (es) and the two-layered nucellus (n). All cells of both the inner (ii) and outer (oi) integuments react with MAC 207. Magnification, $\times 105$. *b*, Representative image taken from a complete longitudinal series through an ovary (fixed ~ 3 days before anthesis) containing eight ovules. There is no evidence of unreactive ovule cells at this time. As the change in MAC 207 expression in the stamen preceded that in the carpel by several days, the anther in the top right corner contains a largely unreactive sporangium. Magnification, $\times 95$.

lineages of a flowering plant from an early stage. The absence of the MAC 207 epitope from globular-stage embryos indicates that the typical sporophytic condition is re-acquired only when the main vegetative organ systems of the sporophyte become established, but then its complete re-expression indicates that the developmental change is fully restored.

We find that the changes described in the MAC 207 epitope

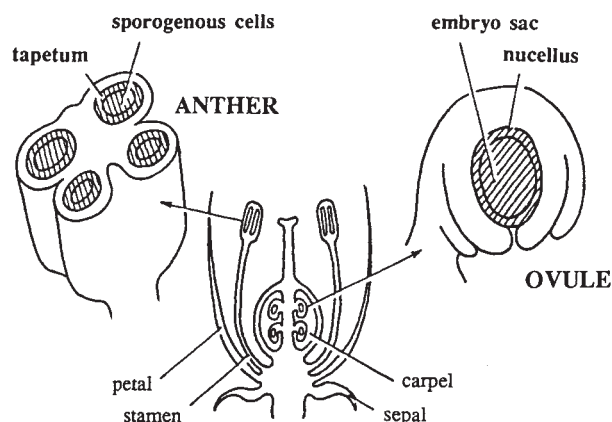


FIG. 4 Schematic median longitudinal section of a flower. The hatching in the enlargements indicates the distribution of MAC 207-unreactive cells.

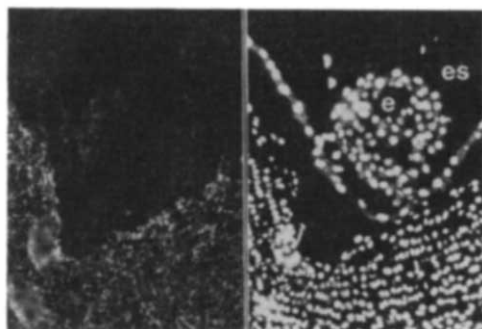


FIG. 5 Median longitudinal section of part of a developing seed (~3 days after fertilization), fixed and labelled as described in Fig. 1 with MAC 207 (left) and DAPI (right). The developing pericarp of the fruit (formerly the integument of the ovule) continues to bind MAC 207, but inside the embryo sac (es), neither the globular 128-cell embryo (e), nor the endosperm following the inner contour of the pericarp (both products of fusion between gametes that do not contain the MAC 207 epitope), fluoresce. Magnification, $\times 150$.

for *Pisum* are essentially similar in another dicot, *Arabidopsis thaliana*, and also in the monocot *Zea mays* (data not shown). We do not yet know whether the MAC 207 epitope is masked in nonreactive cells, whether it is missing from otherwise intact glycoproteins, or whether the entire AGP family is developmentally excluded from the germ tissue. Although immunoblotting of anther preparations indicates that entire glycoproteins are absent (data not shown), each possibility has parallels in animal development⁶. Clearly, a definitive answer to this question can only be obtained from expression studies with the genes encoding the core proteins of the MAC 207-reactive AGPs.

In angiosperm development, we identify a transient stage in which the MAC 207 epitope disappears. This begins in the flower at the time of determination of the sporangial lineages, and ends during embryonic development; during this interval fertilization occurs. We suggest that this developmental 'window' relates to the absence from the plant embryo of a germ lineage³, and may arise specifically as a consequence of it. Certainly, the presence of the MAC 207 epitope in vegetative primordia indicates that neither somatic cell commitment nor cell differentiation depend on its loss, and the uninterrupted presence of the epitope during somatic embryogenesis in *Daucus* (data not shown) implies that the change in AGP expression relates not to embryogenesis *per se*, but specifically to sexual reproduction. A soluble MAC 207-reactive *Daucus* AGP is a lectin that binds β -linked glycans⁵, but it is unlikely that alteration in related extracellular matrix-binding AGPs of the plasma membrane⁷ could be responsible for the deposition of $\beta(1\rightarrow3)$ - rather than $\beta(1\rightarrow4)$ -linked glucans associated with the change from vegetative to sexual phase⁸.

Many genes are likely to be involved specifically with sexual reproduction. Flower-specific complementary DNA clones have recently been isolated from *Lycopersicon*, and other genes have been described in *Arabidopsis* and *Zea* that seem to be involved in directing floral morphogenesis and specifying floral parts⁹. Some of these genes have now been cloned⁹, but none seem to be involved in AGP expression. Rather, we suggest that because the AGPs recognized by MAC 207 form a family of up to 18 abundant, extrinsic, extensively glycosylated species⁵ (forming the equivalent of the animal cell glycocalyx⁷), their developmental exclusion from the male and female sporangia (and gametophytes, with the exception of the vegetative cell) may be an essential prelude to the expression in the gametes themselves of determinants involved with fertilization. Ultrastructural

evidence for gamete-level recognition and preferential fertilization in *Plumbago*¹⁰ illustrates the probable existence of such determinants. □

Received 24 November 1989; accepted 26 February 1990.

1. Arber, A. *Biol. Rev.* **12**, 157-184 (1937).
2. Esau, K. in *Anatomy of Seed Plants* 2nd edn, 375-401 (Wiley, New York, 1977).
3. Walbot, V. *Trends Genet.* **1**, 165-169 (1985).
4. Fincher, G. B., Stone, B. A. & Clarke, A. E. *Rev. Pl. Physiol.* **34**, 47-70 (1983).
5. Pennell, R. I., Knox, J. P., Scofield, G. N., Selvendran, R. R. & Roberts, K. *J. Cell Biol.* **108**, 1967-1977 (1989).
6. Feizi, T. & Childs, R. A. *Biochem. J.* **245**, 1-11 (1987).
7. Roberts, K. *Curr. Op. Cell Biol.* **1**, 1020-1027 (1989).
8. Bell, P. R. *Adv. Bot. Res.* **16**, 55-87 (1989).
9. Drews, G. N. & Goldberg, R. B. *Trends Genet.* **5**, 256-261 (1989).
10. Russell, S. D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6129-6132 (1985).

ACKNOWLEDGEMENTS. We thank P. R. Bell (University College London) and R. S. Poethig (University of Pennsylvania) for comments on the manuscript, and N. J. Brewin (John Innes Institute) for the MAC 207 hybridoma. The research was funded by an EEC Biotechnology Action Programme (R.I.P.).

Regulation of mitosis by cyclic accumulation of p80^{cdc25} mitotic inducer in fission yeast

Sergio Moreno*, Paul Nurse* & Paul Russell†

* ICRF Cell Cycle Group, Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK

† Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, USA

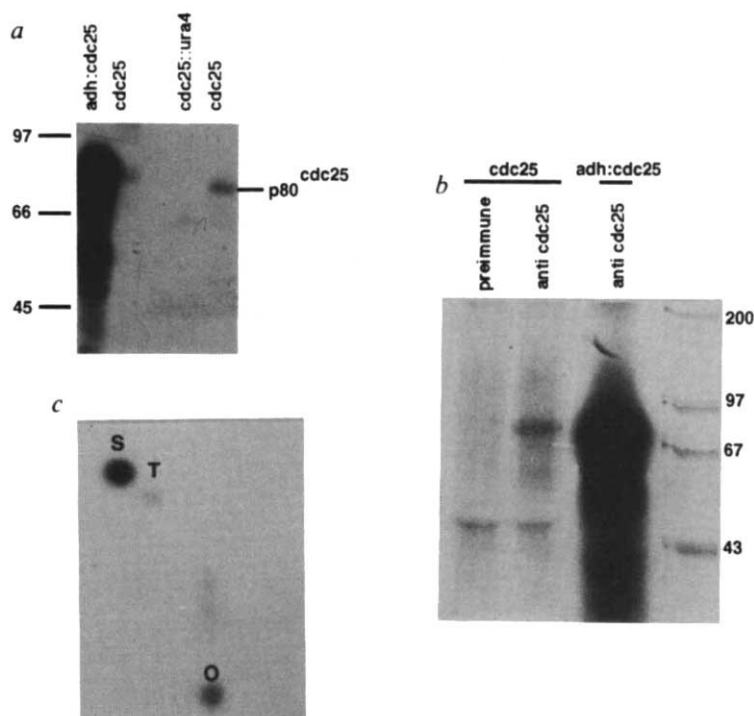
THE coordination of somatic cell division with cell size must be accomplished by the accumulation of mitotic inducers or the dilution, in the course of cell growth, of mitotic inhibitors. In fission yeast (*Schizosaccharomyces pombe*), cell size at mitosis is determined by expression of the *cdc25*⁺ and *nim1*⁺ inducer genes and of the inhibitor gene *wee1*⁺, which between them regulate the M-phase protein kinase p34^{cdc2} (refs 1-7). We now report that both the phosphoprotein product of *cdc25*⁺ (p80^{cdc25}, with apparent relative molecular mass 80,000) and the corresponding messenger RNA increase in concentration as cells proceed through interphase, peaking at mitosis. We propose that the cell-cycle timing of mitosis in somatic cells is regulated by the cyclic accumulation of the *cdc25* mitotic inducer, which on reaching a critical level results in activation of p34^{cdc2} protein kinase. Accumulation of this inducer could play a part in coordinating cell division with growth.

The level of *cdc25*⁺ expression determines cell size at mitosis. A five-fold increase in *cdc25*⁺ gene dose causes ~25% reduction of cell size at division, and there is a greater mitotic advancement in cells in which *cdc25*⁺ transcription is driven by a strong promoter³. Expression of the *cdc25*⁺ gene is normally required at entry into mitosis, for p34^{cdc2} protein dephosphorylation, and for kinase activation^{2,3,6-9}, although it is not necessary for mitosis in cells lacking the *wee1*⁺ inhibitor^{2,3}. Therefore the *cdc25*⁺ gene product acts as an important rate-limiting inducer regulating the onset of mitosis in wild-type cells. To further investigate the *cdc25*⁺ gene product, we produced polyclonal antisera against *cdc25* protein made in *Escherichia coli* (Fig. 1 legend). Immunoblot analysis showed that the *cdc25* antisera specifically recognized fission yeast protein species migrating with an apparent relative molecular mass of 80,000 (*M_r* 80K) on SDS-PAGE (Fig. 1a). The *cdc25*⁺ gene product made in *E. coli* migrated with the same apparent *M_r* (data not shown). The 80K protein was absent in *cdc25*-deletion strains (*cdc25::ura4*⁺) and present at greatly increased amounts in strains overproducing *cdc25* (*adh::cdc25*⁺). The *cdc25*-specific antibodies could also

† To whom correspondence should be addressed.

FIG. 1 Immunoblot and immunoprecipitation analysis of *cdc25*⁺ gene product. *a*, *S. pombe* soluble protein extracts from wild-type (*cdc25*), *cdc25* overproducer (*adh:cdc25*) and *cdc25* deletion (*cdc25::ura4*) were electrophoresed on an 8% SDS-PAGE gel, electroblotted, and probed with anti-*cdc25* antibody followed by ¹²⁵I-labelled protein A. The positions and sizes of protein *M_r* markers are indicated on the left. *b*, *S. pombe* wild-type (*cdc25*) or *cdc25* overproducer (*adh:cdc25*) strains were metabolically labelled with ³²P₀₄, and protein extracts made in RIPA buffer (10 mM sodium phosphate, pH 7, 1% Triton X-100, 1% deoxycolate, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 4 µg ml⁻¹ leupeptin, 1 mM PMSF) were immunoprecipitated with *cdc25* antibody or preimmune antibody. Immunoprecipitates were electrophoresed in an 8% SDS-PAGE gel, and the dried gel was exposed to film. *c*, Phosphoaminoacid analysis of p80^{*cdc25*} from *cdc25*⁺ cells labelled with ³²P₀₄ *in vivo*. S, phosphoserine; T, phosphothreonine; O, origin.

METHODS. Protein production, purification, rabbit injections, bleeds and affinity purification were performed as described²⁵. Antibodies were affinity-purified against p80^{*cdc25*} coupled to cyanogen bromide-activated Sepharose CL4B. Protein extracts for immunoblotting were made by breaking cells with glass beads in lysis buffer (20 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 20 µg ml⁻¹ leupeptin, 20 µg ml⁻¹ pepstatin, 20 µg ml⁻¹ aprotinin). The extract was collected, diluted 1:1 with 2 × protein sample buffer, and boiled for 5 min. Immunoblots were performed as described²⁵ using ¹²⁵I-labelled protein A. The filters were analysed by autoradiography and quantified with a LKB Ultrascan XL laser densitometer. Signals were normalized by scanning a duplicate SDS-PAGE gel that had been stained with Coomassie blue. Cells were labelled with ³²P₀₄ as described²⁶. Phos-



phoamino-acid analysis was performed as previously described²⁷. The *cdc25*-deletion strain had the genotype *cdc25::ura4*⁺. *cdc2-3w ura4-D18 leu1-32*; the *cdc25* overproducer strain had the genotype *adh:cdc25*⁺ (*ura4*⁺) *ura4-D18 leu1-32*.

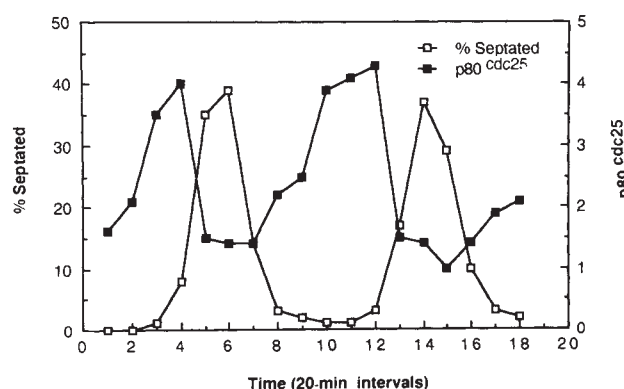


FIG. 2 Oscillation of p80^{*cdc25*} level during the cell cycle. The smallest cells in a log-phase wild-type (972) culture grown at 29 °C were obtained by elutriation centrifugation and inoculated into the original growth medium depleted of cells. After 25 min samples were taken at 20-min intervals. The plot shows the septation index (□) and p80^{*cdc25*} level (■) as determined by densitometric scans of the immunoblot and normalization to total protein loaded in each lane, as determined by densitometric scan of Coomassie blue-stained duplicate gel. The p80^{*cdc25*} immunoblot is shown below the plot. **METHODS.** For analysis of protein levels in synchronous cultures, 4 l of wild-type (972) cells were grown at 29 °C in YES medium²⁶ to a density of 4 × 10⁶ cells ml⁻¹. Cells were separated according to size by elutriation centrifugation, using a Beckman JE 5.0 elutriator rotor in a Beckman J6M/E centrifuge. The cell-depleted (conditioned) medium was retained and incubated at 29 °C. After 3.8 l cells were loaded into the rotor, the cells in the elutriation chamber were flushed with the conditioned growth medium. One litre of cells was eluted at a density of 1.5 × 10⁶ cells ml⁻¹. These cells were returned to a shaking waterbath incubator at 29 °C, and the first sample was taken after 25 min when cells were in mid-G2 phase. Synchrony was monitored by septation index determined by counting at least 100 cells using dark-field optics, and by measuring cell numbers with a Coulter counter (Model ZM, Coulter Electronics).

immunoprecipitate p80^{*cdc25*} from fission yeast. Using extracts made from cells metabolically labelled with ³²P₀₄, anti-*cdc25* antibodies specifically precipitated an 80K protein (Fig. 1b), which was extremely prominent in immunoprecipitates made from cells overproducing *cdc25*. Phosphoaminoacid analysis of the protein from wild-type cells revealed the major phosphoaminoacid as phosphoserine, with phosphothreonine as a minor species (Fig. 1c).

To investigate whether p80^{*cdc25*} levels change as cells progress towards mitosis, we monitored p80^{*cdc25*} in cultures undergoing synchronous rounds of cell division. Synchronous cultures were produced by centrifugal elutriation, a method that avoids potential artefacts caused by induced synchrony protocols. Samples were taken during two cell cycles, and the level of p80^{*cdc25*} was monitored by immunoblotting, normalizing to the amount of protein loaded in each lane. The p80^{*cdc25*} level oscillated with a single peak in each cell cycle, rising to a maximum shortly before the septation index peaked, near the time of the G2/M transition (Fig. 2). The cellular concentration of p80^{*cdc25*} rapidly decreased as the septation index began to increase at about the end of the M phase.

We next determined whether the change in p80^{*cdc25*} level is mediated by an increase in the level of *cdc25*⁺ mRNA. Using a synchronous culture produced with the elutriator rotor, we measured the level of *cdc25*⁺ mRNA by northern-blot analysis, and normalized it to *ura4*⁺ mRNA (Fig. 3). The level of *cdc25*⁺ mRNA oscillated with a single peak in each cell cycle. The maximum correlated closely with the peak of p80^{*cdc25*}. These results, together with the observation that increased *cdc25*⁺ mRNA levels result in increased levels of p80^{*cdc25*} (Fig. 1), indicate that the accumulation of p80^{*cdc25*} during interphase is achieved by a regulatory mechanism controlling the level *cdc25*⁺ mRNA during the cell cycle. We do not yet know whether this control operates by regulating the rate of *cdc25*⁺ transcription or mRNA stability.

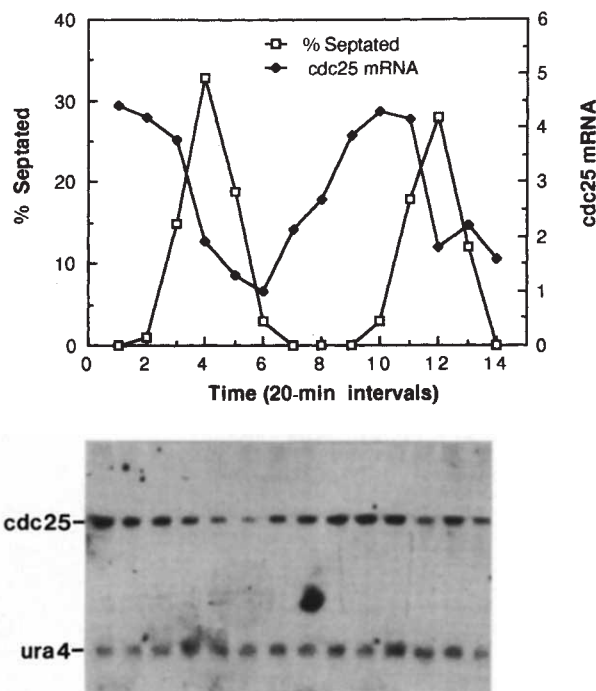


FIG. 3 Periodic expression of *cdc25*⁺ mRNA. A synchronous culture was made as described in the legend to Fig. 2; RNA extracts were made at 20-min intervals, and the level of *cdc25*⁺ mRNA was monitored by northern-blot analysis. The plot shows the septation index (□) and *cdc25*⁺ mRNA level (◆). The *cdc25*⁺ mRNA level was normalized to *ura4*⁺ mRNA. The northern blot is shown below the plot.

METHODS. RNA was prepared from synchronized cells as previously described²⁸. After glyoxal denaturation, 15 µg RNA samples were electrophoresed in an agarose gel, blotted to GeneScreen Plus (Dupont NEN) using 10 × SSC medium, hybridized, and washed, according to the manufacturers instructions. The *cdc25*-specific probe was a 1.9-kilobase (kb) fragment containing the exact *cdc25* open reading frame. The control probe was made using the 1.9 kb *Hind*III fragment containing *ura4*⁺ (ref. 29).

To characterize better what happens to p80^{*cdc25*} as cells proceed through G2 and mitosis, we performed immunoblot experiments using extracts made from *cdc25-22* and *cdc2-33* cells, which arrest in late G2, and *cdc13-117* cells, which arrest in mid-mitosis^{6,10}. In all cases accumulation of p80^{*cdc25*} was about 4-to-8-fold higher than that of asynchronous cultures (Fig. 4), indicating that p80^{*cdc25*} continues to accumulate in cells blocked both in late G2 and mitosis, possibly because the decrease that normally takes place at the end of mitosis cannot occur in these mutants. Decreasing the temperature of the *cdc25-22* culture to 25 °C, resulted in an increase in the level of p80^{*cdc25*} by about another two-fold as cells entered M phase and then a decrease as cells proceeded through mitosis, although the decrease was slower than that seen in the selection synchronous culture (Fig. 2). These patterns could be influenced by the fact that a temperature-sensitive *cdc25* mutant was used to synchronize these cells. Similar results were obtained on decreasing the temperature of a *cdc2-33* culture to 25 °C (data not shown). These data show that p80^{*cdc25*} levels are highest in late G2 and early M phase, and then fall as cells complete mitosis.

We have demonstrated here that the level of *cdc25*⁺ encoded mitotic inducer, an 80K phosphoprotein, increases as cells approach mitosis. The accumulation of p80^{*cdc25*} is achieved by a regulatory mechanism that increases the level of *cdc25*⁺ mRNA during interphase. The timing of mitosis is quite sensitive to the level of *cdc25*⁺ expression³. Therefore, fission yeast p80^{*cdc25*} has two of the essential attributes of 'division initiator proteins' that have been postulated to drive mitosis in somatic cells: its level contributes to the rate at which cells progress through G2 to

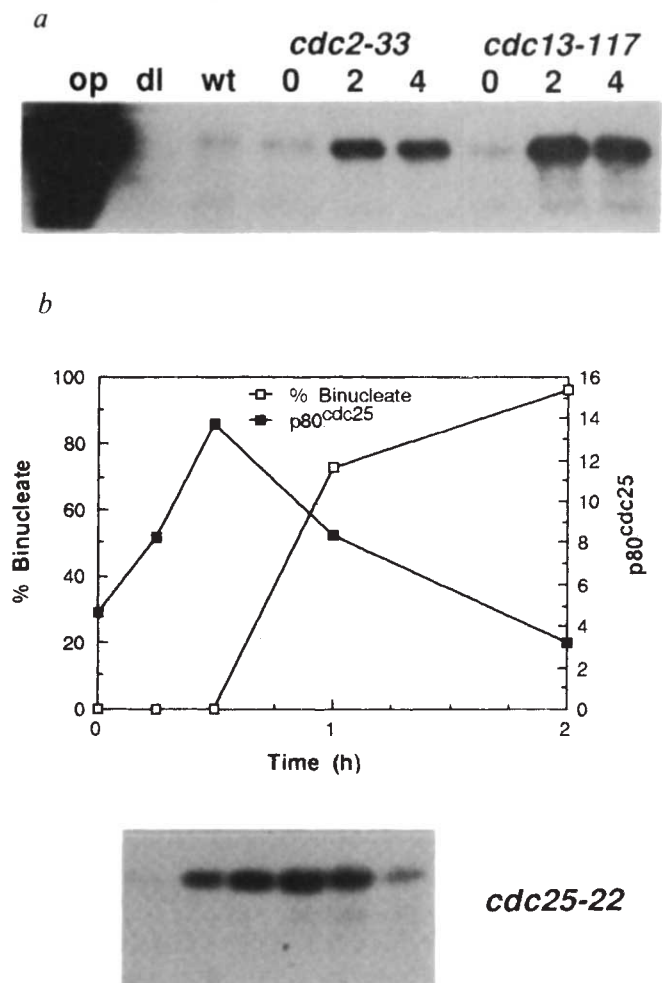


FIG. 4 The level of p80^{*cdc25*} rises and falls as cells proceed through mitosis. a, Level of p80^{*cdc25*} was monitored during the cell division cycle by immunoblotting using *cdc25* affinity-purified antibody. The *cdc2-33* and *cdc13-117* mutants were grown at the permissive temperature (25 or 29 °C, respectively) and then shifted to 35.5 °C. Numbers indicate hours at 35.5 °C. Abbreviations: op, *cdc25* overproducer *adh::cdc25*⁺; dl, *cdc25* deletion *cdc25::ura4*⁺; wt, wild type. b, Log-phase *cdc25-22* culture was shifted to 35.5 °C for 3 h, and then shifted back to 25 °C. Samples were taken at the indicated time points and processed for immunoblotting with anti-*cdc25* antibody. The plot shows the level of p80^{*cdc25*} (■), relative to the amount of p80^{*cdc25*} in the asynchronous culture at 25 °C, at times after the shift back to 25 °C. Also shown is the percentage of binucleate cells (□) as monitored by 4,6-diamidino-2-phenylindole staining, which in this case also includes cells that have completed mitosis and formed a septum. Below the plot is the corresponding *cdc25* immunoblot with the following samples shown left to right: cells growing at 25 °C before shifting to 35.5 °C; 3 h at 35.5 °C; 0.25, 0.5, 1 and 2 h after shift to 25 °C.

initiate mitosis, and its cellular concentration increases as cells grow. The periodic accumulation of p80^{*cdc25*} mitotic inducer, integrated with the activities of other rate-limiting mitotic inducers and inhibitors, must play an important part in determining the timing of mitotic initiation in fission yeast.

The *cdc25*⁺-encoded mitotic inducer has a role in the mitotic control of growing cells that differs from that of cyclins, another class of proteins that are required for activation of p34^{*cdc2*} protein kinase^{6,7}, and that accumulate during interphase and are destroyed during mitosis in a variety of cell types¹¹⁻¹⁷. Studies in fission yeast^{10,18} and *Drosophila*¹⁷ indicate that the levels of cyclins are not rate-limiting for mitotic initiation in somatic cells. In contrast to the situation in somatic cells, cyclins could be rate-limiting M-phase inducers in *Xenopus* eggs and in extracts¹²⁻¹⁴, although alternative interpretations are possible in

these systems¹⁹. Dephosphorylation of p34^{cdc2} could regulate p34^{cdc2} kinase and maturation-promoting factor (MPF) activation in *Xenopus*^{20,21} and starfish oocytes²², but it is not known whether a cdc25 homologue is involved in these processes. This possibility should be considered in view of the recent identification of budding yeast and *Drosophila* genes that have sequence homology to cdc25⁺ (refs 23, 24). Both genes function in mitotic control and are able to complement cdc25-22 when expressed in fission yeast. Furthermore, zygotic mRNA expression of the *Drosophila* cdc25⁺ homologue *stg*, seems to be cell-cycle regulated²⁴. This indicates that periodic expression of cdc25⁺ homologues could be a general feature of the mitotic control in somatic cells.

The biochemical mechanism by which p80^{cdc25} promotes activation of p34^{cdc2} protein kinase remains unknown. It is of interest that p34^{cdc2} becomes dephosphorylated at tyrosine and threonine residues when p80^{cdc25} function is restored to cells after shifting a cdc25^{ts} mutant from a restrictive to a permissive temperature⁹. Dephosphorylation of p34^{cdc2} is coincident with its activation as a protein kinase. These observations, together with mutagenesis studies showing that alteration of the p34^{cdc2} tyrosine phosphorylation site enhances its activity as a mitotic inducer, indicate that the p34^{cdc2} is activated by dephosphorylation. We suggest that it is the accumulation of p80^{cdc25} to a critical level that results in p34^{cdc2} dephosphorylation, possibly either by activating phosphatases or inhibiting kinases. □

Received 11 December 1989; accepted 29 January 1990.

1. Nurse, P. *Nature* **256**, 547-551 (1975).
2. Fantes, P. *Nature* **279**, 428-430 (1979).
3. Russell, P. & Nurse, P. *Cell* **45**, 145-153 (1986).
4. Russell, P. & Nurse, P. *Cell* **49**, 559-567 (1987a).
5. Russell, P. & Nurse, P. *Cell* **49**, 569-576 (1987b).
6. Moreno, S., Hayles, J. & Nurse, P. *Cell* **58**, 361-372 (1989).
7. Booher, R. N., Alfa, C. A., Hyams, J. S. & Beach, D. H. *Cell* **58**, 485-497 (1989).
8. Hagan, I. M. & Hyams, J. S. *J. Cell Sci.* **89**, 343-357 (1988).
9. Gould, K. L. & Nurse, P. *Nature* **342**, 39-45 (1989).
10. Hagan, I. M., Hayles, J. & Nurse, P. *J. Cell Sci.* **91**, 587-595 (1988).
11. Evans, T., Rosenthal, E., Youngblom, J., Kistel, D. & Hunt, T. *Cell* **33**, 389-396 (1983).
12. Murray, A. W. & Kirschner, M. W. *Nature* **339**, 275-280 (1989).
13. Murray, A. W., Solomon, M. J. & Kirschner, M. W. *Nature* **339**, 280-286 (1989).
14. Murray, A. W. & Kirschner, M. W. *Science* **246**, 614-621 (1989).
15. Minshull, J., Blow, J. & Hunt, T. *Cell* **56**, 957-968 (1989).
16. Pines, J. & Hunter, T. *Cell* **58**, 833-846 (1989).
17. Lehner, C. F. & O'Farrell, P. *Cell* **56**, 957-968 (1989).

18. Booher, R. & Beach, D. *EMBO J.* **7**, 2321-2327 (1988).
19. Nurse, P. *Nature* **344**, 503-508 (1990).
20. Dunphy, W. G. & Newport, J. W. *Cell* **58**, 181-191 (1989).
21. Gautier, J., Matsukawa, T., Nurse, P. & Maller, J. *Nature* **339**, 626-629 (1989).
22. Labbe, J. C., Picard, A., Peaucellier, G., Cavadore, J. C., Nurse, P. & Doree, M. *Cell* **57**, 253-263 (1989).
23. Russell, P., Moreno, S. & Reed, S. I. *Cell* **57**, 295-303 (1989).
24. Edgar, B. A. & O'Farrell, P. H. *Cell* **57**, 177-187 (1989).
25. Harlow, E. & Lane, D. *Antibodies* (Cold Spring Harbor Laboratory, New York, 1988).
26. Moreno, S., Klar, A. & Nurse, P. *Meth. Enzym.* (in the press).
27. Cooper, J. A., Sefton, B. M. & Hunter, T. *Meth. Enzym.* **99**, 387-402 (1983).
28. Aves, S. J., Durkacz, B. W., Carr, A. & Nurse, P. *EMBO J.* **4**, 457-463 (1985).
29. Bach, M. L. *Curr. Genet.* **12**, 527-534 (1987).

ACKNOWLEDGEMENTS. We thank Haleh Akhavan-Niaki, Carol Featherstone, Tamar Enoch, Kathy Gould, Jim Hicks, Chris Norbury, Steve Reed, Aresa Toukdarian and Curt Wittenburg for comments and advice, Harriet Hemmingsen and Jacqueline Hayles who kindly provided the RNA samples and northern blot. S.M. was supported by a fellowship from Consejo Superior de Investigaciones Científicas. This work was supported by the NIH and the ICRF.

Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription

Russell C. Hirsch^{*†}, Joel E. Lavine[‡], Lung-ji Chang^{*}, Harold E. Varmus^{*†} & Don Ganem^{*§||}

Departments of ^{*}Microbiology and Immunology, [†]Biochemistry and Biophysics, [‡]Pediatrics and [§]Medicine, University of California Medical Center, San Francisco, California 94143, USA

ALL reactions involving reverse transcription of RNA are segregated from the cytosol within a subviral particle or capsid composed of the major capsid protein, the polymerase and the RNA template¹. A key step in the formation of these particles is the selective encapsidation of the RNA template. Although an important general feature of the reverse transcription pathway, encapsidation has been carefully studied only for retroviruses²⁻⁵. We have now examined the encapsidation reaction in a family of enveloped DNA viruses that replicate by reverse transcription—the hepatitis B viruses (hepadnaviruses). Our results indicate that the hepadnaviral polymerase (P) gene product is required for RNA packaging, and that the encapsidation function of the enzyme can be separated from its DNA polymerase activity. To our knowledge, this is the first description of a role for polymerase gene products in this step of the reverse transcription pathway.

Figure 1 shows the coding organization and transcription map of the duck hepatitis B virus (DHBV), the avian hepadnavirus that we used in this study. Three open reading frames (*orfs*) are evident: C, encoding the core (or nucleocapsid) protein; P,

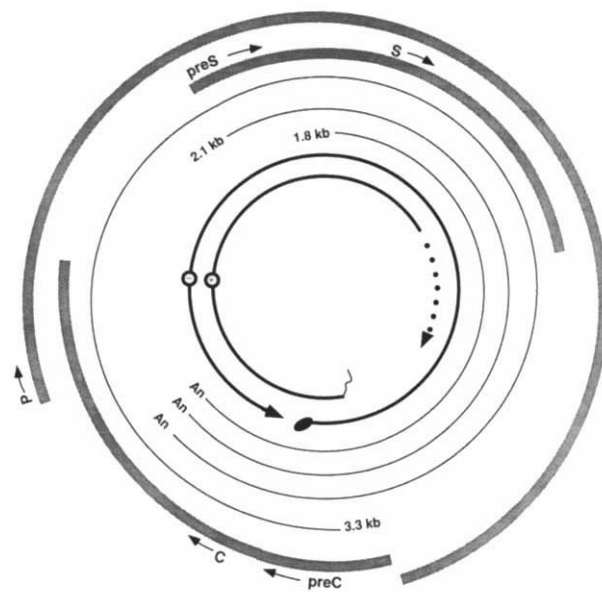


FIG. 1 Genetic and transcriptional map of DHBV. The partially double-stranded DNA genome of DHBV is represented by the inner circles. The 5' end of the minus (-) strand is marked by a solid oval indicating the linkage to a terminal protein. The 5' end of the plus (+) strand, marked by a wavy line indicating attachment to an oligoribonucleotide, is found at a fixed position, whereas the position of the 3' end is variable as indicated by the dotted line. The three thinner lines surrounding the DNA genome correspond to the major transcripts seen in infected cells: the 2.1-kb mRNA encoding pre-S antigen, the 1.8-kb mRNA encoding S antigen, and the 3.3-kb RNA serving both as the template for reverse transcription and as the mRNA encoding core (C) antigen and possibly polymerase (P). The stippled bars indicate the *orf* for the preS/S, C and P genes. An, denotes the mRNA poly(A) tail.

|| To whom correspondence should be addressed.

encoding the polymerase; and preS/S, encoding the viral surface (or envelope) glycoproteins. After entry of the virus into the host, the viral genome is transcribed into two classes of RNAs⁶: subgenomic transcripts (of 1.8 and 2.1 kilobases (kb)), which are the messenger RNAs for the surface glycoproteins, and genome-length (3.3 kb) RNAs. The genomic RNAs serve as both mRNAs (for core protein and potentially for polymerase) and as templates for reverse transcription. Only the genomic RNAs are encapsidated into subviral cores (ref. 7; R.C.H. and D.G., unpublished data); after the encapsidation of the genomic RNA (together with polymerase) in the cytoplasm, reverse transcription occurs to generate the viral DNA genome.

The proteins governing the packaging of viral RNA into subviral cores are unknown, as are the features of the genomic RNA that dictate its selective encapsidation. To define the gene products required for encapsidation, we examined mutant DHBV genomes bearing lesions that inactivate individual viral coding regions for their ability to package genomic RNA. We focused our attention on P gene mutants, because preS/S proteins are not components of nucleocapsids, and C⁻ mutants cannot form cores. To assay for RNA packaging, we transfected Huh7 hepatoma cells with wild-type or P⁻ DHBV DNA. From one-half of each sample of transfected cells, we isolated total cellular poly(A)⁺ RNA. From the other half, we purified cytoplasmic core particles and extracted their nucleic acids. We then determined the genomic RNA present in equal portions of each preparation by primer-extension analysis.

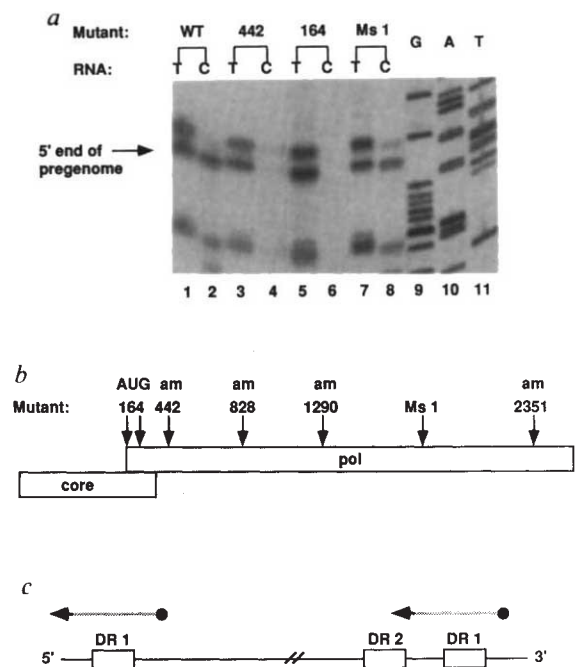
Figure 2a (lanes 1 and 2) shows the results of such an analysis of cells transfected with wild-type DHBV DNA. These cells contain abundant quantities of pre-genomic RNA in the pool of total cell poly(A)⁺ RNA (lane 1), as judged by the appropriate primer-extension product (arrow). (The other bands in this primer extension reaction resulted from strong stops to reverse transcription; because sequences complementary to the primer

are present in both of the terminal redundancies on the RNA (Fig. 2c), extension from the downstream priming site generates strong stops that are both longer and shorter than the signature band defining the 5' end of the RNA.) Examination of the encapsidated RNA from the same cells (lane 2) revealed that a large amount of genomic DHBV RNA is found within core particles. From other experiments in which we quantitated viral RNAs in the two pools using nuclease-protection assays (see Fig. 4), we estimate that about 50–60% of the total genomic RNA is encapsidated.

We next performed similar studies of a variety of P gene mutants (depicted in Fig. 2b). The results for two representative null mutants of *orf* P are shown: mutant 442, containing a frameshift mutation in the P gene coding region just 3' to the C–P overlap; and mutant 164, in which both of the two in-frame AUG codons of *orf* P are changed to ACG. Both mutants are defective in viral DNA synthesis and are noninfectious⁸. Mutants 442 (lane 3) and 164 (lane 5) were transcribed correctly, accumulating normal levels of properly initiated genomic RNA in the total cellular RNA pool (Fig. 2a). But when we examined the encapsidated RNA from cells transfected with these mutants, we could barely detect genomic RNA (lanes 4 and 6 for mutants 442 and 164, respectively), indicating that P⁻ mutant genomes are packaging-defective. Quantitation of this and other experiments (Fig. 4) indicates that only 2–3% of the total genomic RNA of mutant 442 is encapsidated. This effect is not due to failure to synthesize core protein or core particles, because mutant 442 can complement a C⁻P⁺ genome efficiently in a co-transfection assay (see below). We obtained identical results with three additional chain-terminating mutants (828, 1290 and 2351) depicted in Fig. 2b (data not shown). So the failure of these RNAs to be packaged is not due to disruption of a putative packaging signal, but to the inactivation of the P gene product itself.

FIG. 2 Primer-extension analysis of total and encapsidated pre-genomic RNA in transfected cells. **a**, Cells were transfected with either wild-type (WT) DHBV DNA (lanes 1 and 2) or the indicated mutant DNA (lanes 3–8). From one-half of each sample of transfected cells, total cellular poly(A)⁺ RNA was isolated (lanes labelled T), and from the other half cytoplasmic cores were purified and the nucleic acid extracted (lanes labelled C). The genomic RNA present in equal portions of each fraction was quantitated by primer extension of an end-labelled oligonucleotide primer complementary to the 5' portion of the C *orf*. Lanes 9–11, sequence ladder of WT DHBV genome generated with the same primer used for primer extension. The band representing the 5' end of the WT and mutant pre-genomic RNA is indicated by an arrow. **b**, Schematic representation of the C and P *orfs*. Mutant 164 is a missense mutant in which the first two AUG codons (nucleotides 171 and 300) of the P gene have been changed to ACG (ref. 8). Mutant 442 is a frameshift mutant in which the deletion of a T at nucleotide 424 generates a unique *Pst*I site and generates a termination codon in the P reading frame 10 nucleotides downstream of the frameshift mutation⁸. Mutants 828, 1290 and 2351 are chain-terminating mutants containing a synthetic *Xba*I linker with stop codons in all three reading frames cloned into unique *Aat*II, *Kpn*I and *Nco*I sites, respectively. Mutant Ms1 is a missense mutant bearing two base changes engineered into the Tyr-X-Asp-Asp sequence motif conserved in all reverse transcriptases. Abbreviation: am, amber mutation. **c**, Schematic representation of the strategy for primer extension. Primer extension of genomic RNA was performed using a 5' end-labelled synthetic oligonucleotide primer (indicated by a solid circle) complementary to the 5' end of the C gene and to the same sequence in the downstream terminal redundancy of the RNA. Extension from the downstream priming site generates strong stops that are both longer and shorter than the authentic 5' end of the genomic RNA (a). The intensity of the bands corresponding to the strong stop that is longer than the authentic 5' end of genomic RNA is greatly decreased in the pool of encapsidated RNA relative to the pool of total cellular poly(A) RNA, owing to the 3'-to-5' degradation of genomic RNA by RNase H during reverse transcription. DR, denotes direct repeat sequences involved in viral DNA synthesis.

METHODS. Transfection, nucleic acid preparation, primer extension and electrophoresis were performed as described^{10,19}. Plasmid constructions were



performed with the European strain of DHBV²⁰. Plasmids p442 and p164 are tandem dimers of P gene mutants 253 and AUG 1,130, respectively, previously described by Chang *et al.*⁸ Mutants 828, 1290 and 2351 were constructed by cutting the European strain of DHBV with *Aat*II, *Kpn*I and *Nco*I, respectively, generating blunt ends by treatment with T4 DNA polymerase, and inserting a 14-nucleotide synthetic *Xba*I stop-codon linker with T4 DNA ligase. The construction of Ms1 will be described elsewhere (L.-J.C. *et al.*, manuscript in preparation).

Although all chain-terminating mutations in *orf P* are packaging-defective, not all P-gene lesions affect packaging. Figure 2a also illustrates a packaging study of a missense P mutant (Ms1) bearing two base changes engineered into a sequence encoding the motif Tyr-Met-Asp-Asp conserved in all reverse transcriptases. This mutant is completely defective in viral DNA synthesis (L.-J. C., manuscript in preparation). But RNA from this genome is packaged at wild-type efficiency (Fig. 2a, lanes 7 and 8). This implies that a polymerase functional for DNA synthesis is not required for genomic RNA encapsidation.

We next asked if the encapsidation defect of P^- mutants can be complemented by provision of P gene products in *trans* from a co-transfected P^+ genome. Although we could demonstrate such complementation, it was surprisingly inefficient. Figure 3 shows a representative example of results with encapsidated progeny DNA from cells co-transfected with C^+P^- and C^-P^+ genomes. In this experiment, the two parental genomes were marked with distinct restriction sites so that the origin of the progeny DNA genomes could be determined by restriction mapping (see Fig. 3 legend).

As expected, transfection of each mutant alone resulted in no progeny DNA synthesis (lanes 1 and 2). When the mutants were co-transfected, encapsidated product DNA was generated (lane 3), in agreement with earlier results^{8,9}. But cleavage of

this DNA with either *Pst*I (lane 4), *Nsi*I (lane 5) or both *Nsi*I and *Pst*I (lane 6) revealed that most of the DNA molecules lacked recognition sites for both enzymes and hence were of the C^-P^+ genotype. Over-exposure of this film (data not shown) revealed a small amount of progeny C^+P^- DNA, indicated by the presence of the 2.4-kb *Nsi*I-*Pst*I product fragment; we estimate that these genomes represent about 5–10% of the products. As shown previously¹⁰, similar results were obtained when we used wild-type DNA to complement the P^- genome in an analogous experiment (lanes 7–9). Again, restriction analysis of the complementation products revealed that most of the encapsidated progeny DNA originated from the P^+ genotype. Together, these studies indicate that the provision of P function in *trans* by wild-type genomes is considerably less efficient than provision of core antigen.

Because of the peculiar behaviour of the P gene product in complementation experiments in which viral DNA synthesis is scored, we examined the RNA encapsidation function of the P protein for complementation in *trans*. To this we developed an assay for encapsidated RNA based on RNase protection methods that can distinguish wild-type pre-genomic RNA from that of the P^- mutant. Our strategy for this is illustrated in Fig. 4b and detailed in its legend; in this assay, wild-type RNA generates two product fragments of 430 and 300 nucleotides, whereas P^- RNAs protect a 730-nucleotide species.

We transfected Huh7 cells with either mutant or wild-type DNA, or both, and examined total or encapsidated RNA by RNase protection (Fig. 4). Examination of the total cellular poly(A)⁺ RNA revealed that both wild-type (lane 4) and mutant (lane 6) genomes were abundantly transcribed, and that their RNAs generated protected fragments of appropriate size. As expected, transfection of the P^- genome alone resulted in virtually no packaged RNA (lane 5). When co-transfected, both wild-type and mutant genomes were equally represented in the total RNA (lane 8); but, assembled cores again contained predominantly the wild-type genomic RNA and only small quantities of mutant RNA (lane 7).

These data indicate that products of the DHBV P gene are involved not only in reverse transcription but also in the selective packaging of the genomic RNA that serves as the template for this reaction. This role for P gene products in this step reaffirms that hepadnaviral core assembly differs importantly from cognate events in retroviruses. Retroviral RNA packaging is mediated principally by capsid (*gag*-encoded) proteins^{2,3}; some evidence indicates that polymerase (*pol*) gene products may not be required, as certain *pol*⁻ mutant genomes are efficiently packaged in murine retroviruses¹¹. Another difference concerns how reverse transcriptase is directed to the core particle. Retroviral polymerase is expressed as a polyprotein fused to the capsid polypeptides. After incorporation into subviral particles (presumably mediated by its *gag* domain), the *gag-pol* polyprotein is proteolytically processed to liberate the mature reverse transcriptase^{12,13}. Despite its economy and simplicity, this biosynthetic pathway is not conserved in the hepadnaviruses. DHBV polymerase is not made by synthesis of a C-P polyprotein, but rather by *de novo* translation initiated at the first AUG codon of the P gene^{8,9}. This indicates that the mechanism used by the DHBV polymerase for incorporation into subviral cores could be different from that used by its retroviral counterpart.

How does the encapsidation of genomic RNA occur? Presumably, the P protein is involved either directly or indirectly in the selection of genomic viral RNA for encapsidation. The P gene product could bind directly to this RNA, or noncovalent interactions with core antigen (or other proteins) could form a complex that mediates this recognition. In either case, this RNA recognition event must signal or trigger incorporation of the RNA-protein complex into subviral cores. How it does so remains unknown, but there are several possibilities. If the recognition event generates a core-P protein-RNA complex, the complex could be incorporated into cores through interactions of its C

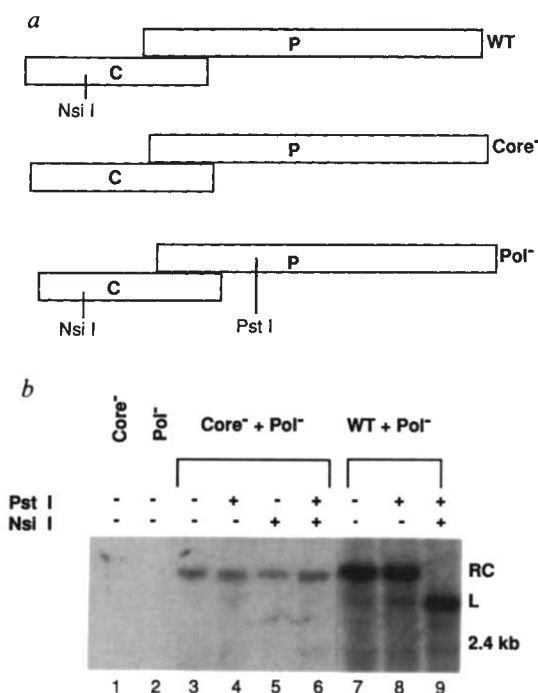


FIG. 3 Complementation of core-negative and polymerase-negative genomes. *a*, Schematic representation of wild-type (WT), C^+P^- , and C^-P^+ genomes. The WT DHBV genome has a unique *Nsi*I site in the C gene, and contains no *Pst*I sites. In the C^-P^+ genome ('core⁻'), ablation of the unique *Nsi*I site in the C gene causes a frameshift and premature termination of the C protein. In the C^+P^- genome ('pol⁻', mutant 442), deletion of a single base just 3' to the C-P overlap creates a unique *Pst*I site and results in premature termination of the P protein. *b*, Southern blot of the replicative forms of viral DNA synthesized in Huh7 cells after transfection. Huh7 cells were transfected with C^-P^+ alone (lane 1), C^+P^- alone (lane 2), C^-P^+ with C^+P^- (lanes 3–6), or WT with C^+P^- (lanes 7–9). DNA was extracted 5 days later from purified cores and examined by restriction-endonuclease cleavage and Southern blotting with ³²P-labelled DHBV DNA as a probe. In lanes 6 and 9, digestion with *Pst*I and *Nsi*I generated a 2.4-kb fragment only from encapsidated C^+P^- progeny DNA. RC and L, positions of relaxed circular and linear DHBV DNA, respectively.

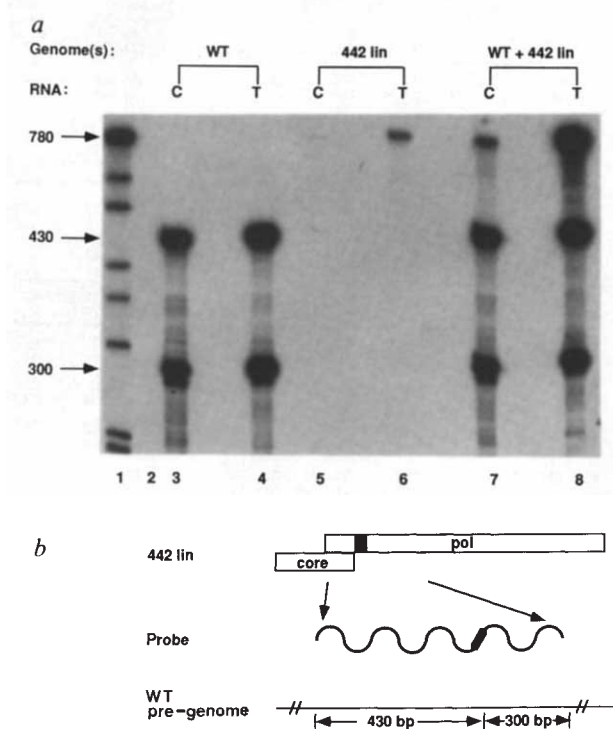


FIG. 4 RNase protection analysis of total and encapsidated pregenomic RNA in transfected cells. Cells were transfected with wild-type (WT) DHBV DNA or mutant DNA, or both, and from one-half of each sample of transfected cells, total cellular poly(A)⁺ RNA was isolated, and from the other half, cytoplasmic cores were purified and the nucleic acid extracted. The genomic RNA present in equal portions of each preparation was quantitated by nuclease protection as described in *b*. Lane 1, full-length (780 nucleotides) undigested probe with relative molecular mass (M_r) standards ($M_r \times 10^{-3}$); lane 2, probe digested in absence of added sample RNA; lanes 3, 5 and 7, fragments protected by core RNA (C) from cells transfected with WT DNA, P⁻ mutant 442 lin DNA (442 lin), or WT and 442 lin DNA, respectively; lanes 4, 6 and 8, fragments protected by total cellular poly(A)⁺ RNA (T) from cells transfected with WT, 442 lin, and WT and 442 lin, respectively. The numbers to the left of lane 1 represent the size of the full-length probe (780 nucleotides) and the size of the protected fragments (430 and 300 nucleotides) generated by WT DHBV RNA. *b*, Nuclease-protection assay for distinguishing WT pre-genomic RNA from P mutant pre-genomic RNA. For this assay, plasmid p442 was modified to yield plasmid p442 lin by insertion of a 14-nucleotide linker bearing multiple stop codons at the unique *Pst*I site of plasmid p442. A 730-nucleotide fragment of p442 lin spanning the linker was cloned into an SP6 transcription vector (to generate p442 linP) and used to generate a uniformly labelled RNA probe complementary to this region. The probe was annealed to genomic RNA, and single-stranded regions were digested by RNase. This probe was essentially completely protected (about 30 nucleotides of plasmid polylinker would have been digested) by P mutant genomic RNA; WT DHBV RNA should protect fragments of 430 and 300 nucleotides.

METHODS. For probe generation 0.5 μ g linearized p442 linP was transcribed in a 20- μ l reaction mixture containing 40 mM Tris buffer, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 2 μ g BSA, 0.5 mM each ATP, GTP and CTP, 0.05 mM UTP, 1 μ l [α -³²P]UTP (400 Ci mmol⁻¹), 20 U RNasin, and 20 U SP6 RNA polymerase (Promega). After 1-h incubation at 37 °C, 1 μ l (1 U μ l⁻¹) of RNase-free DNase was added, and the incubation was continued for another 15 min before addition of phenol-chloroform, and extraction. For RNase mapping, 200,000 Cherenkov-radiation c.p.m.-equivalent of probe was hybridized with sample RNA in a 30- μ l hybridization mixture containing 40 mM HEPES buffer, pH 7.9, 400 mM NaCl and 1 mM EDTA. The nucleic acids were heated to 85 °C for 10 min and then allowed to cool to 65 °C for 3 h. After hybridization, digestion was performed at 30 °C for 1 h by adding 200 μ l ice-cold 300 mM NaCl, 10 mM Tris buffer, pH 7.5, 5 mM EDTA, 40 μ g ml⁻¹ RNase A, and 400 U ml⁻¹ RNase T1 to each hybridization mixture. The solution was then adjusted to 150 μ g ml⁻¹ proteinase K and 0.6% SDS, and incubated at 37 °C for 15 min. After extraction, precipitation and denaturation, the samples were electrophoresed through a 6% polyacrylamide-8 M urea gel.

moiety with assembling C-protein subunits. Alternatively, P protein (or C-P complex) binding to RNA could alter the secondary structure of the RNA so that a sequence required for packaging is exposed.

It is curious that there is an apparent preference for action in *cis* of the P-gene packaging function. This *cis* preference is consistent with models of packaging involving either (1) coupling of encapsidation to pre-genome translation, with packaging being mediated by nascent polypeptide chains interacting with their genomic RNA messengers as they emerge from the ribosome, or (2) the production of limiting amounts of P protein so that conditions favouring encapsidation occur only near the site of P translation. Consistent with the latter idea is the recent demonstration that vectors expected to overproduce P protein complement P⁻ genomes more efficiently than do wild-type genomes (T.-T. Wu, L. D. Condeelis and W. Mason, personal communication; R. Bartenschlager and H. Schaller, personal communication). Further experiments will be required to distinguish between these possibilities. Hepadnaviral P protein is not the only example of a gene product displaying asymmetric complementation: similar phenomena have been observed for the transposase proteins of the bacterial transposons Tn5 and Tn10 (refs 14, 15).

These and other studies of hepadnaviruses^{16,17} and retroviruses^{2,18} illustrate the striking diversity of routes by which different genetic elements have solved common problems associ-

ated with reverse transcription. A detailed understanding of these mechanisms should inform studies of the replicative strategies of other less-well-characterized elements that depend on reverse transcription. □

Received 16 November 1989; accepted 30 January 1990.

1. Fuetterer, J. & Hohn, T. *Trends biochem. Sci.* **12**, 92-95 (1987).
2. Meric, C. & Spahr, P.-F. *J. Virol.* **60**, 450-459 (1986).
3. Gorelick, R. J., Henderson, L. E., Hanser, J. P. & Rein, A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8420-8424 (1988).
4. Mann, R., Mulligan, R. C. & Baltimore, D. *Cell* **33**, 153-159 (1983).
5. Adam, M. A. & Miller, A. D. *J. Virol.* **62**, 3802-3806 (1988).
6. Buscher, M., Reiser, W., Will, H. & Schaller, H. *Cell* **40**, 717-724 (1985).
7. Enders, G. H., Ganem, D. & Varmus, H. E. *J. Virol.* **61**, 35-41 (1987).
8. Chang, L.-J., Pryciak, P., Ganem, D. & Varmus, H. E. *Nature* **337**, 364-367 (1989).
9. Schlicht, H.-J., Radziwill, G. & Schaller, H. *Cell* **56**, 85-92 (1989).
10. Lavine, J., Hirsch, R. & Ganem, D. *J. Virol.* **63**, 4257-4263 (1989).
11. Shields, A., Witte, O. N., Rothenberg, E. & Baltimore, D. *Cell* **14**, 601-609 (1978).
12. Dickson, C., Eisenman, R., Fan, H., Hunter, E. & Teich, N. in *RNA Tumor Viruses Ch. 6* (Cold Spring Harbor Laboratory, New York, 1982).
13. Eisenman, R. & Vogt, V. *Biochem. biophys. Acta* **473**, 187-239 (1978).
14. Berg, D. in *Mobile DNA* 185-210 (American Society of Microbiology, New York, 1989).
15. Morisato, D., Way, J., Kim, H. & Kleckner, N. *Cell* **32**, 799-807 (1983).
16. Bartenschlager, R. & Schaller, H. *EMBO J.* **7**, 4185-4192 (1988).
17. Molnar-Kimber, K. L., Summers, J., Taylor, J. M. & Mason, W. S. *J. Virol.* **45**, 165-172 (1983).
18. Prats, A. C. et al. *EMBO J.* **7**, 1777-1783 (1988).
19. Hirsch, R., Colgrove, R. & Ganem, D. *Virology* **167**, 136-142 (1988).
20. Sprengel, R., Kuhn, C., Will, H. & Schaller, H. *J. Med. Virol.* **15**, 323-333 (1985).

ACKNOWLEDGEMENTS. We thank Drs Silvija Staprans, Roland Russnak and Titia de Lange for helpful discussions, and Drs W. Mason and H. Schaller for permission to cite their unpublished data. This work was supported by the NIH.

western blotting using an antibody (AbP1) against peptide P1. Cells transfected with pBTF3a overexpressed a 27K protein corresponding to purified BTF3 (Fig. 2a, compare lane 4 with lanes 1 and 2), whereas pBTF3b-transfected cells overexpressed a 22K protein (Fig. 2a, lane 5). Both the 27K and 22K proteins were detected at a much lower level in the control cells (Fig. 2a, compare lanes 3–5 in the lower panel). The experiments shown in Fig. 2b (compare experiment 1 with experiment 2 and experiment 3) show that the AbP1 antibody is specific for BTF3, as it can remove BTF3 protein and activity from a partially purified HeLa-cell BTF3 fraction (see legend to Fig. 2 for details). Whole-cell extracts of pBTF3a-transfected cells were then successively fractionated on a heparin-Ultrogel column (results not shown), a DEAE-5PW column (Fig. 3a) and a glycerol gradient (results not shown), and the fractions tested for BTF3 activity in a transcription system lacking BTF3. As expected, BTF3 activity (T) from BTF3a-transfected cells cofractionated with the immunoreactive 27K protein (WB), whereas with the amount of extracts that are used, the endogenous BTF3 activity was too low to be detected in pSG5-transfected cells (Fig. 3, compare a and b). By contrast, no BTF3 activity could be detected in the pBTF3b-transfected cells, although AbP1 revealed the presence of the 22K protein (Fig. 3c, lanes 9–14; and data not shown). Also BTF3 activity cosedimented with the 27K protein, and not the 22K protein, when mixed fractions from pBTF3a- and pBTF3b-transfected cells were sedimented

through a glycerol gradient (Fig. 3d). The first N-terminal 44 amino acids of BTF3a are therefore indispensable for its transcriptional function. We note that both BTF3a and BTF3b sedimented as 20K–30K proteins, indicating that they exist as monomeric proteins in solutions⁶.

RNA pol II(B) was mixed with partially purified BTF3a or BTF3b, and sedimented on glycerol gradients (Fig. 4). RNA pol II was also sedimented alone as a control (Fig. 4a). For the

FIG. 1 Sequence of BTF3a and BTF3b cDNAs and the predicted BTF3a and BTF3b proteins. a and b, Sequences of the cDNA inserts and the deduced amino-acid sequence of BTF3a and BTF3b. The amino-acid sequences corresponding to peptides 1 to 4 (see text) are underlined (P1–P4 in a). In b, dotted lines indicate identity of sequences between BTF3b and BTF3a. Stop codons are boxed. Amino acids are marked relative to position 1 in the ORF. ▲, Point of divergency between BTF3a and BTF3b sequences. c, Schematic representation of BTF3a and BTF3b cDNA sequences (upper numbers) and deduced amino-acid sequences (lower numbers). The N-terminal (from residues 1 to 96) and the C-terminal (from residues 160 to 206) domains are highly hydrophilic, and are either basic (particularly from residues 63 to 79, black box) or acidic (last 30 amino acids, hatched box). The central domain of BTF3a (residues 96 to 160) is highly hydrophobic (dotted box) and leucine-rich (residues 142 to 160). d, Predicted (Bioexplore, Biostructure, Strasbourg) amphipathic α -helix in the BTF3a region from residues 143 to 154. ●, hydrophobic amino acids (single-letter code: L (leu), 143; M (Met), 146; L, 147; I (Ile), 150; L, 151; L, 154).

METHODS. BTF3 microsequencing. The BTF3 factor present in the glycerol gradient-purified fraction 1 (GG1; ref. 6) was further purified by 10% SDS-PAGE. The 27K BTF3 polypeptide was eluted overnight at room temperature in 1 ml of 50 mM Tris-HCl buffer, pH 7.9, containing 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl and 0.1% SDS. The eluted polypeptide (400 pmol) was subjected to reverse-phase HPLC (Brownlee aquapore RP300; 7 μ ; 1 $\times$ 250 mm; gradient, 0–60% acetonitrile/0.1% TFA in 120 min; flow rate, 0.05 ml min⁻¹). The 0.2 ml peak fraction was then concentrated under vacuum, resuspended in 50 μ l of 100 mM Tris-HCl buffer, pH 8.5, containing 1 M urea, and digested with 1 μ g trypsin (Boehringer) at 37 °C for 12 h. The hydrolyzed peptides were resolved by reverse-phase HPLC and microsequenced (Applied Biosystems model 477A protein sequencer). Complementary DNA cloning. The MCF-7 human breast cancer cell (λ gt11) cDNA library was as previously described³³ and the HeLa cell library was a gift of Dr S. Green. Phage from each library ($\sim 2 \times 10^5$) were transferred to 150 mm nitrocellulose filters in duplicate and screened using a set of degenerate ³²P-end-labelled oligonucleotide probes (probes 1–4) designed³⁴ from the amino-acid sequence of peptides P1, P2, P3 and P4 (a). Hybridization using the different probes ($\sim 1 \times 10^6$ c.p.m. ml⁻¹) was performed at 54–60 °C for 12 h in 5 $\times$ SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0), 10 $\times$ Denhardt's solution (0.2% Ficoll, 0.2% polyvinyl pyrrolidone, 0.2% serum albumin) and denatured salmon sperm DNA (50 μ g ml⁻¹). Filters were washed twice for 15 min at 37 °C in 3 $\times$ SSC containing 0.1% SDS, dried and exposed to Kodak X-ray film with an intensifying screen at –80 °C for 12 h. The first screening was performed with probe 1. Positive plaques were rescreened with the other oligonucleotide probes. Complementary DNA inserts from positive phage clones were subcloned into either the M13mp18 sequencing vector or Genescribe-Z vector pTZ18U (USB). Clones pB3a and pB3b which hybridized with probes 1–4 and probes 1, 2 and 4, respectively, were sequenced on both strands using the dideoxynucleotide chain termination method.

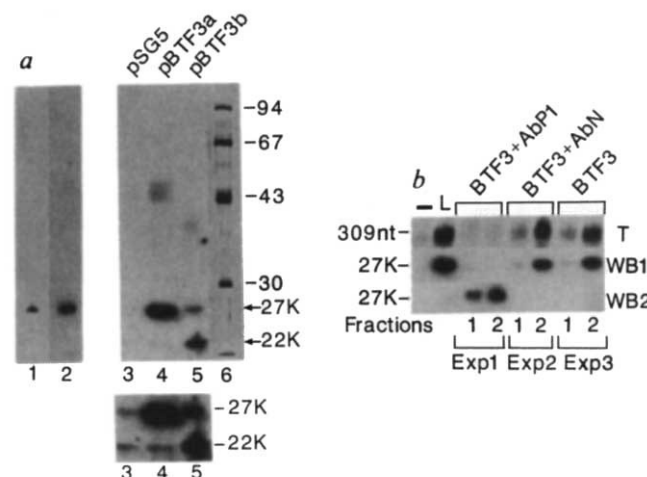


FIG. 2 Immunoreactivity of purified and cloned BTF3 factor with the antibody preparation AbP1. a, Western-blot analysis. Lane 1, silver staining of a 10% SDS-PAGE of HPLC-purified BTF3 (20 ng). Purified BTF3 (lane 2) and whole-cell extracts from HeLa cells transfected (see legend to Fig. 3) with either the parental expression vector pSG5 as a control (lane 3), pBTF3a (lane 4) or pBTF3b (lane 5), were reacted with AbP1 antibody after SDS-PAGE. The lower panel (lanes 3–5) displays a threefold longer exposure. Arrows point to the 27K and the 22K polypeptides which react with AbP1. b, Protein A-Sepharose chromatography. Glycerol gradient-purified (GG1) BTF3⁶ was incubated (overnight, 4 °C) in PBS (pH 7) with either 10 μ l (20 μ g) ammonium sulphate-precipitated AbP1 (Exp 1), 5 μ l (20 μ g) ammonium sulphate-precipitated AbN preimmune serum (Exp 2), or alone (Exp 3). The samples were then separately absorbed on a 150- μ l protein A-Sepharose column (Pharmacia), washed with PBS, and elution performed with a glycine-HCl buffer (pH 3). The first two flow-through fractions (150 μ l each, fractions 1 and 2) were tested for both immunoreactivity with AbP1 (WB1) and transcriptional activity (T) using an *in vitro* transcription assay lacking BTF3, but containing all the general transcription factors in addition to RNA pol II^{5,6}. Fractions eluted at pH 3 were also analyzed by western blotting (WB2). The specific Ad2MLP 309-nucleotide (nt) run-off transcript¹⁴ and the 27K polypeptide interacting with AbP1 are indicated. L and – lanes, control transcriptional and immunoreactivity assays with and without purified BTF3, respectively.

METHODS. AbP1 antibody preparation and Western-blot analysis. Peptide 1 (P1 in Fig. 1a) and its ovalbumin conjugate were from Neosystem (France). After collection of preimmune serum (AbN), rabbits were injected with 100 μ g ovalbumin-conjugated P1 in 1 ml of PBS emulsified with an equal volume of Freund's complete adjuvant. Booster injections (incomplete Freund's adjuvant) were administered 2, 4 and 6 weeks later. Blood samples were collected 2 weeks after the third booster injections. The corresponding serum (AbP1) reacted specifically (ELISA) with P1 and conjugated ovalbumin P1 at dilutions $> 1:1,000$ (data not shown). Samples containing BTF3 were electrophoresed (10% SDS-PAGE) and electroblotted onto nitrocellulose sheets for 1 h at 25 °C in a 50 mM Tris-HCl (pH 7.4) buffer containing 0.56% glycine and 10% methanol³⁵. The blots were first incubated with 3% milk powder in PBS for 1 h at 25 °C, and then 1 h at 37 °C with ammonium sulphate-precipitated (40%) AbP1 (2 mg ml⁻¹) diluted 250-fold in PBS buffer. After extensive washing with PBS containing 0.05% Tween-20 at 25 °C, the nitrocellulose blots were incubated for 1 h at 24 °C with radiolabelled protein A (Amersham), rewashed extensively, and exposed overnight. *In vitro* transcription assay. An Ad2MLP EcoRI–SalI template (50 ng) from plasmid pM34, 4 μ g STF (DEO.35), 2.5 μ g BTF1 (DEO.25), 2.2 μ g BTF2 (SP0.35), partially purified HeLa RNA pol II (0.01 U) and aliquots of the various BTF3-containing fractions, were incubated with nucleotide triphosphates and processed for analysis of specific transcription from Ad2MLP as described⁶.

mixture of RNA pol II and BTF3a (Fig. 4b), one peak of activity capable of complementing a transcription assay lacking both RNA pol II and BTF3 was observed, coincident with the presence of the 27K protein BTF3a (WB) and the peak of RNA pol II activity (T) (Fig. 4a and b, compare lanes 10–14; we note in a the absence of BTF3 in the purified RNA pol II and the presence of excess BTF3a 27K protein in the top region of the

gradient in b (WB, lanes 1–6)). For the mixture of RNA pol II and BTF3b, no transcriptional activity could be detected, but some of the 22K protein cosedimented with RNA pol II (Fig. 4c, compare T with WB). Therefore, both BTF3a and BTF3b can form complexes with RNA pol II. Further sedimentation experiments (Fig. 4d) showed that BTF3a was 3-to-4-fold more efficient than BTF3b at interacting with RNA pol II, indicating that the extra 44 N-terminal residues of BTF3a are involved in binding to RNA pol II.

Analysis of the BTF3a sequence failed to reveal any of the known DNA-binding motifs (such as a helix-turn-helix or a zinc-finger motif; see refs 21, 22) and protein-protein interaction motifs (for example, a leucine-zipper; see ref. 23). The hydrophobicity profile of BTF3a shows that the N- and C-terminal domains are highly hydrophilic, and either basic or acidic (Fig.

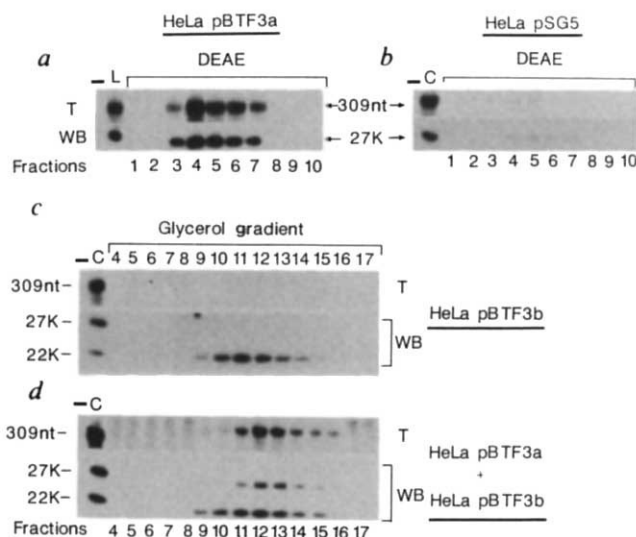


FIG. 3 BTF3 activity of clone BTF3a and BTF3b expressed in HeLa cells. Whole-cell extracts from HeLa cells transfected with either pBTF3a, pBTF3b or the parental expression vector pSG5 (used as a control) were chromatographed onto a heparin-Ultrogel column. Aliquots of the transcriptionally active and/or immunoreactive fractions derived from pBTF3a- and pBTF3b-transfected cells, and their corresponding pSG5 fractions, were pooled and applied onto DEAE-5PW columns (a and b, and data not shown). The relevant DEAE-eluted fractions were then sedimented through a 10–30% glycerol gradient (c, and data not shown). d, Glycerol-gradient sedimentation of an equimolar mixture (based on immunoreactivity) of DEAE fractions derived from HeLa cells transfected with either pBTF3a or pBTF3b. Transcription (T) and western-blot immunoreactivity (WB) assays were as in Fig. 2. Lanes –, control assays lacking BTF3; lanes L, assays of aliquots of samples loaded onto the columns or glycerol gradients; lanes C in b, control assay performed with heparin/Ultrogel-purified BTF3 prepared from pBTF3a-transfected cells. Lanes C in c and d, control assay performed with a mixture of heparin/Ultrogel-purified BTF3a and BTF3b.

METHODS. The BTF3a cDNA *EcoRI* insert subcloned into pTZ18U was digested with *HinfI* (which cuts at nucleotides 165 and 759, see Fig. 2a), and the resulting 0.6-kilobase (kb) fragment was ligated at both ends with the *EcoRI*-*HinfI* linker, 5'-AATTCTAACCACCATGCGACGGACAGGCGACCCGCTCAGGCTG-3'. The BTF3b cDNA *EcoRI* insert subcloned into pTZ19R was digested with *PstI* and *EcoRI* (which cut at nucleotide 283 and 930, respectively; see Fig. 2b), and the resulting 0.65-kb fragment was ligated with the *EcoRI*-*PstI* linker, 5'-AATTCTAACCACCATGAAAGAAACAATCATGAACCA-GGAAAACTCGCCAACTGCA-3'. Both linkers contain the Kozak consensus sequence²⁰. The two cDNA fragments were then inserted into the pSG5 expression vector³⁶, yielding pBTF3a and pBTF3b, respectively. HeLa cells (10^6 per dish) were transfected with 20 μ g either pBTF3a, pBTF3b or pSG5 expression vector (used as a control) using the calcium phosphate method. After 16 h, cells were washed with DMEM medium and incubated for an additional 4 h in the presence of 2.5% FCS. Whole cell extracts (from 4×10^7 HeLa cells) were prepared and applied onto an 8-ml heparin-Ultrogel column equilibrated in buffer A. Adsorbed proteins were eluted with a 0.24–0.60 M KCl gradient. After dialysis against buffer B (Tris-HCl buffer (20 mM), pH 7.6, KCl (50 mM), EDTA (1 mM), DTT (2 mM), glycerol (20%)), the active heparin-Ultrogel fractions derived from pBTF3a-transfected cells, and the corresponding fractions from pSG5- and pBTF3b-transfected cells, were applied onto 1.5-ml DEAE-5PW columns, and the adsorbed proteins eluted with KCl. The relevant pooled DEAE 0.05–0.20 M KCl fractions were then sedimented through 10–30% glycerol gradients for 18 h at 4 °C and 60,000 r.p.m. (SW60 rotor).

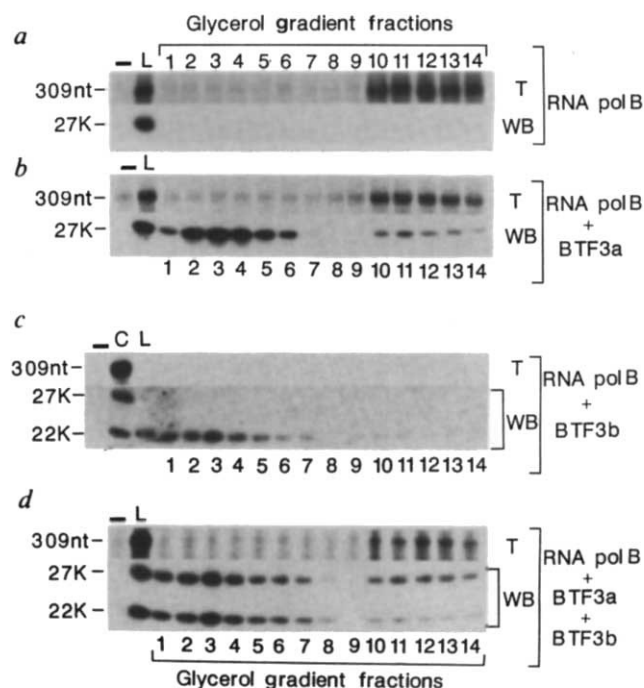


FIG. 4 Interaction of BTF3a and BTF3b with RNA pol II. (B) RNA pol II (0.4 U, DE1M fraction further purified onto a heparin-Ultrogel column as described in ref. 4) was incubated for 10 min at 24 °C, alone (a) or with either 250 μ l BTF3a (b), 250 μ l BTF3b (c) or 250 μ l equimolar mixture (as estimated from immunoreactivity) of BTF3a and BTF3b (d) in 50 mM Tris-HCl buffer, pH 8, containing 20 mM KCl, 0.1 mM EDTA, 5% glycerol, before sedimenting through a 10–30% glycerol gradient for 5 h at 60,000 r.p.m. BTF3a and BTF3b were DEAE fractions derived from pBTF3a- and pBTF3b-transfected HeLa cells as described (Fig. 3, a and data not shown). Transcription assays (T) were performed as described in Fig. 2. a, Transcriptional assay mixture contained all of the necessary components for transcription from Ad2MLP except RNA pol II; lanes – and L, control assays without or with added RNA pol II, respectively. b, Transcriptional assay mixture was as in a, but BTF3 was omitted; Lane – complete transcription assay mixture lacking RNA pol II and BTF3; lane L, aliquot of the mixture loaded onto the gradient. c, Transcriptional assay mixture was as in b; lane C, control assay containing all components necessary for Ad2MLP transcription and aliquots of partially purified BTF3a and BTF3b; lane L, aliquot of the mixture loaded onto the gradient. Lane –, as in b. d, Transcriptional assay mixture as in b; lane L, aliquot of the material loaded onto the gradient; lane –, as in b. In all panels, western blotting with AbP1 (WB) was as described in Fig. 2. The 27K and the 22K polypeptides corresponding to BTF3a and BTF3b, respectively, are indicated. Transcription and western-blot assays were performed with 10–15 μ l or 100–150 μ l glycerol-gradient fractions (lanes 1–14), respectively. Only the relevant portions of the autoradiographs are displayed.

1c). The central domain is highly hydrophobic and rich in leucine residues. The Garnier algorithm^{24,25} predicts that residues 143–154 have a high probability of forming a short amphipathic α -helix with only hydrophobic amino acids (mainly leucines) on one side of the helix (Fig. 1d). This central hydrophobic region is a good candidate for the site of interaction with RNA pol II, and possibly other factors such as BTF2⁶. A similar amphipathic helix with the potential to form a coiled-coil structure seems to be responsible for protein interactions in microtubule crosslinking²⁶. No significant amino-acid homology has been found between the sequence of BTF3 (which may be the same factor as TFIIB, see ref. 1) and those of putative transcription factors or RNA pol II subunits which have recently been cloned (factor SII (ref. 27), RAP30 (ref. 28), 23K subunit of RNA pol II (ref. 29)). It is noteworthy that the amino-acid sequences of BTF3 and RAP30 are unrelated, given that others have suggested that these two transcription factors are identical^{1,30,31}. In this respect we note that no transcriptional function has been reported so far for cloned RAP30 (ref. 28). We have, however, found a 44% identity (data not shown) between residues 5–33 of BTF3 and residues 1–29 of R-ras (ref. 32) for which there is no known transcriptional function (data not shown).

Both BTF3a and BTF3b seem to be evolutionarily conserved because they are present in whole-cell extracts of a variety of cells (human MCF-7, BJA/B and HEPG-2 cells; mouse embryonal carcinoma F9 and NIH 3T3 cells; African green monkey CV1 cells; data not shown). The functional significance of BTF3b is unknown. Although inactive with the adenovirus-2 major late promoter (Ad2MLP), it could function with other promoters. But it cannot replace purified BTF3a or purified endogenous BTF3 for initiation of transcription *in vitro* from the adenovirus-2-IVa2 promoter which lacks a TATA box (data not shown). The puzzling observation that whole-cell extract from HeLa cells transfected with the BTF3b expression vector reproducibly resulted in an increase in the level of BTF3a (Fig. 2a, compare lanes 3 and 5) and BTF3 activity (data not shown), suggests that BTF3b is implicated in the regulation of BTF3a expression. The availability of cloned BTF3 cDNAs should help the elucidation of the molecular mechanisms underlying the seemingly complicated formation, function and regulation of the initiation complex that assembles at the promoter. □

34. Lathe, R. *J. molec. Biol.* **183**, 1–12 (1985).

35. Towbin, H., Staehlin, T. & Gordon, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4355 (1977).

36. Green, S., Issmann, I. & Scheer, E. *Nucleic Acids Res.* **16**, 369 (1988).

ACKNOWLEDGEMENTS. We thank Dr S. Green for MCF-7 and HeLa cell cDNA libraries, P. Lepage (Transgène) for help in protein microsequencing, A. Ferrandon, H. Higgs and J. M. Chipoulet for technical assistance, the cell culture group for providing HeLa cells, A. Staub and R. Ruffenach for synthesizing the oligonucleotides, the secretarial staff for preparation of the manuscript, A. Bieger and J. P. Samana for protein structure analysis, and D. Boam, B. Cavallini, M. Gerard, J. Stévenin and J. White for useful discussions and critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Fondation pour la Recherche Médicale and the Association pour la Recherche sur le Cancer. X.M.Z. was supported by the University Louis Pasteur.

Specific interaction of the murine transcription termination factor TTF I with class-I RNA polymerases

Anne Kuhn, Ingrid Bartsch & Ingrid Grummt

Institut für Biochemie, Röntgenring 11, 8700 Würzburg, FRG

THE 18-base-pair sequence element AGGTCGACCAGTACT-CCG (the Sal box) signals termination of mouse ribosomal gene transcription¹. This sequence is recognized by a sequence-specific DNA-binding protein, TTF I, which mediates the termination of transcription by RNA polymerase I (pol I)^{2,3}. Subsequently, the ends of the primary transcripts are trimmed by 10 nucleotides in a sequence-dependent 3'-terminal processing reaction⁴. We have now investigated whether TTF I bound to its target sequence will block elongation by any RNA polymerase by steric hindrance, or whether it is specific for elongation by pol I. The results demonstrate that TTF I directs transcription termination with RNA polymerase I from species as divergent as mouse and yeast, but fails to affect elongation by heterologous polymerases (eukaryotic RNA polymerases II and III, *Escherichia coli* or bacteriophage T3 RNA polymerase). By contrast, purified *lac* repressor bound to its operator sequence stops elongation by both RNA polymerase I and II.

Figure 1a shows the experimental strategy used to study the termination of transcription directed by various RNA polymerases in the presence of affinity-purified transcription termination factor (TTF I). The assay is based on the observation that RNA polymerases preferentially initiate transcription at nicks or single-stranded regions, and therefore allows initiation by purified RNA polymerases in the absence of accessory factors^{5–8}. The template DNA plasmid pCAT554-650 contains 151 base pairs (bp) from the bacterial chloramphenicol acetyltransferase (CAT) gene and 97 bp from the 3'-terminal spacer of mouse ribosomal DNA, including one Sal box terminator element. We linearized this construct with *Bgl*II, and ligated a 14-base oligonucleotide to the free ends yielding 10-base 3' single-stranded tails at both ends. After restriction with *Hind*III, the template was transcribed with purified RNA polymerases in the absence or presence of the murine termination factor TTF I. We initiated the reaction with the dinucleotide UpG, which is complementary to the 3' end of the oligonucleotide and efficiently primes the initiation reaction. The 3' ends of transcripts were mapped by the S1-nuclease technique. Figure 2 shows transcripts synthesized by murine RNA polymerase I. In the absence of TTF I, a 201-nucleotide fragment was detected (RT) which corresponded to the probe DNA that was protected by readthrough transcripts (lane 1). In the presence of TTF I, two shorter transcripts which protected 188 and 178 nucleotides of the probe DNA were generated. The longer transcripts represent terminated RNA chains^{2,3}, whereas the shorter RNA molecules were products of a post-transcriptional processing reaction that removes 10 nucleotides from the primary transcript

Received 22 December 1989; accepted 26 February 1990.

1. Saltzman, A. G. & Weinmann, R. *FASEB J.* **3**, 1723–1732 (1989).
2. Wasyluk, B. *CRC crit. Rev. Biochem.* **23**, 77–120 (1988).
3. Matsui, T., Segall, J., Weil, P. A. & Roeder, R. G. *J. biol. Chem.* **255**, 11992–11996 (1980).
4. Fire, A., Samuels, M. & Sharp, P. A. *J. biol. Chem.* **259**, 2509–2516 (1984).
5. Moncollin, V., Miyamoto, N. G., Zheng, X. M. & Egly, J. M. *EMBO J.* **5**, 2577–2584 (1986).
6. Zheng, X. M., Moncollin, V., Egly, J. M. & Chambon, P. *Cell* **50**, 361–368 (1987).
7. Horikoshi, M. *et al. Nature* **341**, 299–303 (1989).
8. Hahn, S., Buratowski, S., Sharp, P. A. & Guarente, L. *Cell* **58**, 1173–1181 (1989).
9. Eisenmann, D. M., Dollard, C. & Winston, F. *Cell* **58**, 1183–1191 (1989).
10. Schmidt, M. C., Kao, C. C., Pei, R. & Berk, A. G. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7785–7789 (1989).
11. Cavallini, B. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 9803–9807 (1989).
12. Davison, B. L., Egly, J. M., Mulvihill, E. R. & Chambon, P. *Nature* **301**, 680–686 (1983).
13. Sawadogo, M. & Roeder, R. G. *Cell* **43**, 165–175 (1985).
14. Buratowski, S., Hahn, S., Guarente, L. & Sharp, P. A. *Cell* **56**, 549–561 (1989).
15. van Dyke, M. W., Sawadogo, M. & Roeder, R. G. *Molec. cell. Biol.* **9**, 342–344 (1989).
16. Jacob, M. & Galliano, H. *Nucleic Acids Res.* **17**, 2159–2180 (1989).
17. Reed, R. *Genes Dev.* **3**, 2113–2123 (1989).
18. Kozak, M. *Molec. cell. Biol.* **9**, 5134–5142 (1989).
19. Takeishi, K. *et al. J. Biochem.* **106**, 575–583 (1989).
20. Kozak, M. *Nucleic Acids Res.* **15**, 8125–8148 (1987).
21. Klug, A. & Rhodes, D. *Trends biochem. Sci.* **12**, 464–469 (1987).
22. Brennan, R. G. & Matthews, B. W. *J. biol. Chem.* **264**, 1903–1906 (1989).
23. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. *Science* **240**, 1759–1764 (1988).
24. Levin, J. M., Robson, B. & Garnier, J. *FEBS Lett.* **205**, 303–308 (1986).
25. Gibrat, J.-F., Garnier, J. & Robson, B. *J. molec. Biol.* **198**, 425–443 (1987).
26. Lewis, S. A., Ivanov, I. E., Lee, G.-H. & Cowan, J. *Nature* **342**, 498–505 (1989).
27. Hirashima, S., Hirai, H., Nakanishi, Y. & Natori, S. *J. biol. Chem.* **263**, 3858–3863 (1988).
28. Sotma, M., Burton, Z. F. & Greenblatt, J. *Nature* **341**, 410–414 (1989).
29. Pati, U. K. & Weissman, S. M. *J. biol. Chem.* **264**, 13114–13121 (1989).
30. Burton, Z. F., Killeen, M., Sotma, M., Ortolan, L. G. & Greenblatt, J. *Molec. cell. Biol.* **8**, 1602–1613 (1988).
31. Reinberg, D. & Roeder, R. G. *J. biol. Chem.* **262**, 3310–3321 (1987).
32. Lowe, D. G. *et al. Cell* **48**, 137–146 (1987).
33. Green, S. *et al. Nature* **320**, 134–139 (1986).

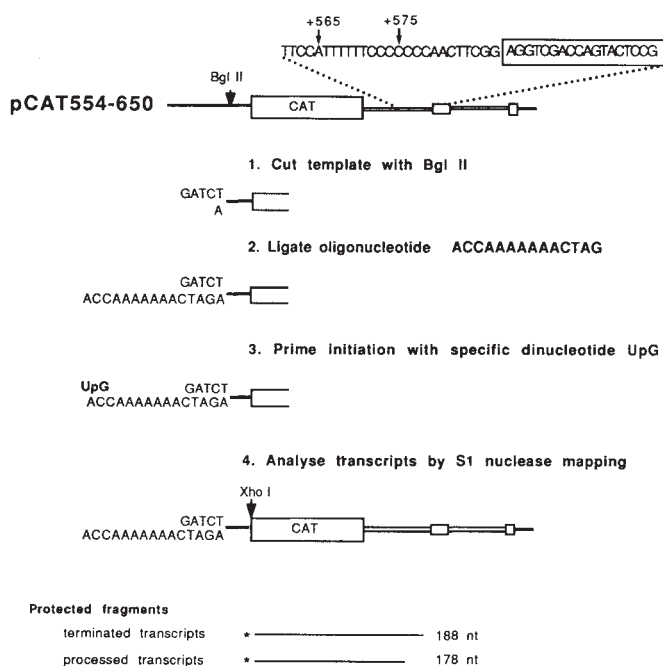


FIG. 1 Schematic representation of the experimental procedure used to generate the 3'-tailed template. Abbreviation: nt, nucleotide.

METHODS. The recombinant plasmid pCAT554-650 contains a 151-bp fragment from the bacterial CAT gene (nucleotides 4,853–5,003 in pSV2-CAT¹⁵) which is fused to a 97-bp fragment from the 3'-terminal spacer region of mouse rDNA (from nucleotide +554 to +650 with respect to the 28S rRNA coding region). The plasmid was cut with *Bgl*II, and a 14-nucleotide oligonucleotide 3'-ACCAAAAAAAGTAG-5' ligated to the cohesive ends. The DNA with two single-stranded extensions was digested with *Hind*III to prevent transcription from the opposite strand. The free oligonucleotides were removed by precipitating the DNA with 7.5% polyethylene glycol 6000 in the presence of 0.9 M NaCl. Tailed template DNA (40 ng) was transcribed in a 25- μ l assay containing 12 mM Tris-HCl buffer, pH 7.9, 0.12 mM EDTA, 85 mM KCl, 5 mM MgCl₂, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, GTP, CTP and UTP, 0.5 mM UpG dinucleotide, and RNA polymerases from different sources as indicated in the figure legends. After incubation for 1 h at 30 °C, the template DNA was digested for 10 min with 0.3 U DNase I; transcripts were purified by phenol-chloroform extraction, and were hybridized for several hours at 50 °C to a 201-bp 3' end-labelled *Xho*I-SalI fragment derived from plasmid pCAT554-650. The hybrids were treated for 60 min at 30 °C with 250 U S1 nuclease, and the nuclease-resistant fragments analysed by electrophoresis on a 6% denaturing gel⁴. Nonterminated readthrough transcripts are of 201 nucleotides. Primary transcripts, which terminate 11 bp upstream of the Sal-box termination signal, yield a 188-nucleotide S1-nuclease-resistant fragment; 3'-terminal processed RNA molecules yield a 178-nucleotide protected fragment. The sequence of the Sal box termination signal and upstream flanking nucleotides encompassing the position of both the termination (+575) and processing site (+565), is shown.

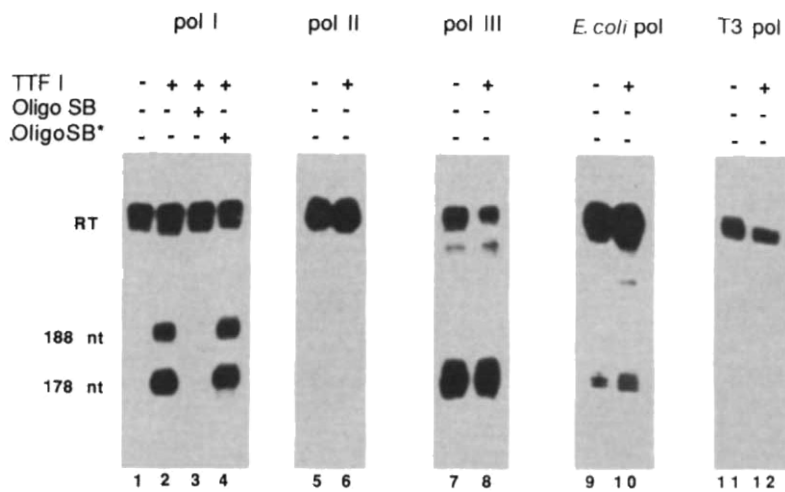
(lane 2)⁴. Neither the 188- or 178-nucleotide band was observed in the presence of 5 pmol Sal box-containing oligonucleotide, which efficiently competes for factor binding (lane 3). By contrast, an oligonucleotide containing a mutant (two base changes) Sal box sequence that excludes TTF I binding² did not affect the termination and subsequent processing reaction (lane 4). This result demonstrates that binding of purified TTF I to the Sal box sequence stops transcribing RNA polymerase I in the absence of any other auxiliary factors.

To investigate whether this transcription termination is the

result of physical blockage of RNA polymerase by the DNA-bound TTF I protein, we assayed heterologous RNA polymerases in the same transcription system. RNA polymerase II from wheat germ (lanes 5 and 6), polymerase III from calf thymus (lanes 7 and 8), *E. coli* RNA polymerase (lanes 9 and 10), and bacteriophage T3 polymerase (lanes 11 and 12) were assayed in the absence and presence, respectively, of the murine termination factor. Clearly, elongation of these heterologous enzymes was not affected by the Sal box/TTF I complex. Transcription with both the wheat germ and the bacteriophage RNA

FIG. 2 S1-nuclease mapping of 3' ends of transcripts synthesized by different classes of prokaryotic and eukaryotic RNA polymerases in the absence and presence of TTF I. The 'tailed' template pCAT554-650 (see Fig. 1) was transcribed with 0.7 U mouse RNA polymerase I (lanes 1–4), 1.5 U wheat germ RNA polymerase II (lanes 5 and 6), 0.9 U calf thymus RNA polymerase III (lanes 7 and 8), 1.3 U *E. coli* RNA polymerase (lanes 9 and 10), or 3.3 U bacteriophage T3 RNA polymerase (lanes 11 and 12) in either the absence or presence of purified factor TTF I. Abbreviation: nt, nucleotide.

METHODS. Purification and functional characterization of murine TTF I was as described previously¹⁶. Template DNA (40 ng) was pre-incubated in the reaction mixture (see legend to Fig. 1) for 10 min at 30 °C with 7.5 μ l either buffer A-100 (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT, 5 mM MgCl₂, 20% glycerol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 100 mM KCl) or purified factor TTF I, before starting the reaction by adding individual RNA polymerases. In two assays containing mouse RNA polymerase I (lanes 3 and 4), 5 pmol of a 35-nucleotide double-stranded oligonucleotide (oligo SB; 5'-GATCCTTCGGAGGTCGACCACTACTCCGGCG-ACA-3') covering the Sal-box sequence (lane 3), or a mutant oligonucleotide (oligo SB*; 5'-GATCCTTCGGAGCGCGACCACTACTCCGGCGACA-3'); lane 4) were included in the reaction mixture to demonstrate the dependence of the termination reaction on the binding of TTF I to its target sequence. After transcription for 60 min at 30 °C, the template was digested with DNase I, and RNA isolated and subjected to S1-nuclease mapping as described in the legend to Fig. 1. The murine pol I was purified from cultured Ehrlich ascites cells ~1,000-fold by chromatography on DEAE-Sephadex (250 mM KCl eluate), Heparin Ultrogel (400 mM KCl eluate), Red-Sephadex (1,000 mM KCl eluate), and Mono Q-FPLC (Pharmacia). The *E. coli* and the phage T3



polymerase were >80% pure. Polymerase II from wheat germ was prepared according to ref. 17, and modified according to ref. 18. Pol III from calf thymus was partially purified by two chromatographic columns. One unit of enzyme activity is defined as the amount of RNA polymerase that incorporates 1 pmol ³H-labelled UMP into acid-precipitable material within 30 min at 37 °C in a nonspecific standard assay containing 7.5 μ g calf thymus DNA as template. The DNA fragments protected by the readthrough transcripts (RT), or by the terminated (188 nucleotide) and processed (178 nucleotide) transcripts, respectively, are indicated.

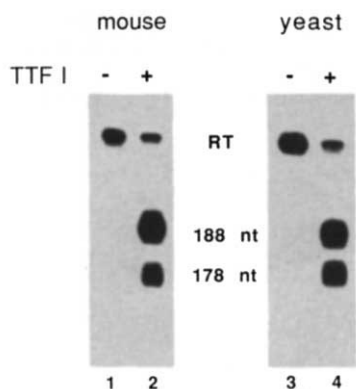


FIG. 3 The murine factor TTF I directs specific termination of transcription by pol I from *S. cerevisiae*. S1-nuclease mapping of transcripts synthesized by RNA polymerase I from mouse (lanes 1 and 2) or yeast (lanes 3 and 4), in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of affinity-purified TTF I. The experimental protocol is identical to that described in the legend to Fig. 2, except that in this experiment 0.6 U mouse enzyme and 0.3 U highly purified yeast enzyme were used. Abbreviation: nt, nucleotide.

polymerases yielded only the readthrough transcripts indicating that the transcribing enzyme bypassed the termination signal, irrespective of whether TTF I was present or not. Also transcription of pol III from calf thymus and of the *E. coli* enzyme was not affected by the mouse factor. But these two enzymes showed factor-independent pausing or termination at the stretch of thymidine residues that coincides with the processing site (see Fig. 1). Conversion of the thymidines (positions +566 to +571) into guanosines abolished the S1 nuclease-resistant band at position +178 (data not shown), indicating that the elongation block was caused by the cluster of thymidine residues. That TTF I was not active when assayed with pol II or III, or with enzymes from bacterial and phage origin, supports a termination mechanism involving specific interactions between pol I and TTF I rather than steric hindrance of the elongation reaction. Because termination of pol I depends on the correct orientation of the Sal box sequence¹, it is reasonable to assume that TTF I is a functionally asymmetric protein, and that binding to its target sequence correctly positions it for specific contact with pol I. As a consequence of this specific protein-protein interaction, the elongation reaction is stopped, and the nascent RNA and the transcribing enzyme are released from the template.

The sequences that direct rDNA transcription termination are very different in various eukaryotes⁹⁻¹³. We therefore studied whether pol I from a distantly related organism is also blocked by the murine factor TTF I. Transcription with pol I from *Saccharomyces cerevisiae* was terminated as efficiently and precisely as was that with mouse pol I (Fig. 3). Surprisingly, the 3'-terminal processing was also observed in the system containing a homogenous preparation of yeast pol I and purified mouse TTF I. Because the processing activity is not associated with the termination factor⁴, this specific nucleolytic activity is probably exerted by pol I itself. The identification of this activity within the multi-subunit pol I enzyme is now in progress.

Next we investigated whether any other protein tightly bound to DNA would also differently affect class-I and -II polymerases. The *lac* operator containing the binding site for the *lac* repressor was placed downstream of the *CAT* gene-marker fragment (pCAT-LO), and transcription by RNA polymerase I and II, respectively, was assayed in the presence and absence of purified *lac* repressor. Both enzymes produced a 404-nucleotide run-off RNA in the tailed template assay (Fig. 4a, lanes 1 and 4). Addition of the *lac* repressor yielded in each case 188-nucleotide transcripts (lanes 2 and 5). This length corresponds to the

distance between the tail and the operator fragment, indicating that elongation stopped near the repressor-operator complex. The terminated transcripts were not observed after adding the inducer isopropylthiogalactoside (IPTG; lanes 3 and 6), indicating that the operator-bound repressor blocks the passage of both pol I and II. To map the site of transcription termination by the *lac* repressor at the nucleotide level, we analysed the 3' ends of the pol II-directed transcripts by the S1-nuclease protection assay. In the sample containing the *lac* repressor and no IPTG (Fig. 4b, lane 2), a 178-nucleotide S1-nuclease resistant fragment appeared which maps the transcription block 22 nucleotides upstream of the centre of the *lac* operator. Transcripts synthesized by pol I map at the same position (data not shown).

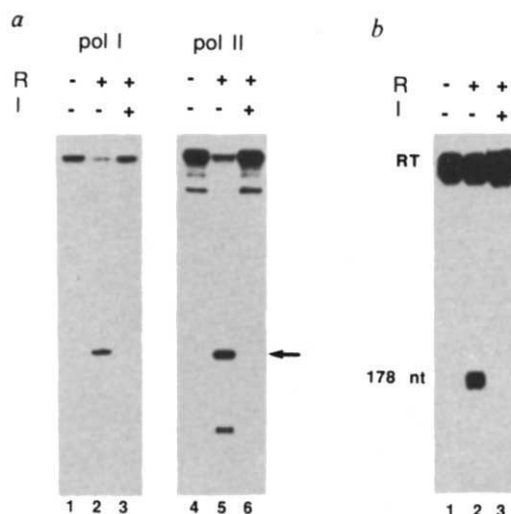


FIG. 4 The *lac* repressor blocks both RNA polymerase I and II. *a*, *In vitro* transcription. Plasmid pCAT-LO was tailed according to the protocol described in the legend to Fig. 1, and was transcribed with either mouse RNA polymerase I (lanes 1-3) or wheat germ RNA polymerase II (lanes 4-6) in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5 and 6) of purified *lac* repressor. R, reactions containing the repressor; I, reactions in which repressor binding was prevented with 0.2 mM IPTG (lanes 3 and 6). Arrow indicates terminated transcripts. The origin of the short RNA molecules (~165 nucleotides) synthesized by pol II in the presence of bound *lac* repressor (lane 5) is unknown. But because these transcripts were not seen in the S1-nuclease mapping experiment (*b*), they probably do not represent prematurely terminated or paused transcripts, but seem to have been transcribed from the opposite DNA strand. *b*, S1-nuclease mapping. The tailed template plasmid pCAT-LO was transcribed by RNA polymerase II as described below, except that the transcription was performed in the presence of 0.66 mM each unlabelled nucleoside triphosphates. The nucleic acids were isolated, treated with DNase I, and the RNA hybridized to a 237-bp *XhoI-EcoRI* fragment derived from plasmid pCAT-LO that had been 3' labelled at the *XhoI* site. After hybridization for 12 h at 48 °C, the hybrids were treated with nuclease S1 and analysed on a 6% sequencing gel. The 178-nucleotide S1 nuclease-resistant DNA fragment corresponds to transcripts that have stopped upstream of the *lac* repressor. Abbreviation: nt, nucleotide.

METHODS. The plasmid pCAT-LO is analogous to plasmid pCAT554-650, except that instead of the 3'-terminal rDNA fragment, a 63-bp *lac* operator fragment was fused to the 151-bp CAT marker fragment. The operator fragment contains a symmetrical 21-bp *lac* operator sequence (AATTGTGAG-CGGATAACAATT)¹⁵ with a centre 210 nucleotides downstream of the artificial initiation site. Tailed template DNA pCAT-LO truncated with *NarI* (40 ng) was pre-incubated for 10 min at 30 °C with 1 µg *lac* repressor (lanes 2, 3, 5 and 6) in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 0.2 mM IPTG in the reaction mixture described in Fig. 1, except that GTP concentration was decreased to 12.5 µM, and 1 µCi [³²P]GTP was added. After pre-incubation, the appropriate RNA polymerase was added and transcription was performed for 5 min. The labelled RNA was isolated and analyzed on a 6% polyacrylamide-urea gel.

Taken together, the different behaviour of different polymerases with respect to termination by TTF I demonstrates that termination with pol I must be more than a physical blockage by a DNA-bound protein. Both termination of pol I transcription and blocking of elongating polymerases by the *lac* repressor is strictly orientation-dependent^{1,14}. This result, together with the finding that none of the heterologous polymerases were affected by DNA-bound TTF I, clearly demonstrates that a protein tightly bound to DNA does not necessarily block transcribing RNA polymerases. We believe that very specific protein-protein interactions between a specific functional domain of RNA polymerase I and TTF I start the sequence of events that terminates transcription and leads to correct 3'-end formation of pre-rRNA. □

Received 5 December 1989; accepted 29 January 1990.

1. Grummt, I., Maier, U., Öhrlein, A., Hassouna, N. & Bachellerie, J.-P. *Cell* **43**, 801–810 (1985).
2. Grummt, I., Rosenbauer, H., Niedermeyer, I., Maier, U. & Öhrlein, A. *Cell* **45**, 837–846 (1986).
3. Kuhn, A., Normann, A., Bartsch, I. & Grummt, I. *EMBO J.* **7**, 1497–1502 (1988).
4. Kuhn, A. & Grummt, I. *Genes Dev.* **3**, 224–231 (1989).
5. Hinkle, D. C., Ruig, J. & Chamberlin, M. J. *J. molec. Biol.* **70**, 197–207 (1972).
6. Dezélee, S., Sentenac, A. & Fromageot, P. *J. J. biol. Chem.* **249**, 5971–5977 (1974).
7. Dezélee, S., Sentenac, A. & Fromageot, P. *J. J. biol. Chem.* **249**, 5978–5983 (1974).
8. Lewis, M. K. & Burgess, R. R. *J. biol. Chem.* **255**, 4928–4936 (1980).
9. Labhart, P. & Reeder, R. H. *J. biol. Chem.* **45**, 431–443 (1986).
10. Labhart, P. & Reeder, R. H. *Molec. cell. Biol.* **7**, 1900–1905 (1987).
11. Kempers-Veenstra, A. E. *et al.* *EMBO J.* **5**, 2703–2710 (1986).
12. Tautz, D. & Dover, G. A. *EMBO J.* **5**, 1267–1273 (1986).
13. Bartsch, I., Schoneberg, C. & Grummt, I. *Molec. cell. Biol.* **7**, 2521–2529 (1987).
14. Deuschle, U. thesis, Univ. Heidelberg (1987).
15. Gorman, C. M., Moffat, L. F. & Howard, B. H. *Molec. cell. Biol.* **2**, 1044–1051 (1982).
16. Bartsch, I., Schoneberg, C. & Grummt, I. *Molec. cell. Biol.* **8**, 3891–3897 (1988).
17. Jendrisak, J. J. & Burgess, R. R. *Biochemistry* **14**, 4639–4645 (1975).
18. Mosig, H., Schäffner, A. R., Sieber, H. & Hartmann, G. R. *Eur. J. Biochem.* **149**, 337–343 (1985).

ACKNOWLEDGEMENTS. For the provision of RNA polymerases we thank M. Riva and A. Sentenac (pol I from yeast), C. Pfeleiderer (pol I from mouse), G. Hartmann (pol II from wheat germ), K. Seifart (pol III from calf thymus), and H. Heumann (*E. coli* RNA polymerase). Purified *lac* repressor was a gift from U. Deuschle and H. Bujard. We thank W. Hädel for the preparation of cell extracts, A. Smid for help in isolating TTF I, and G. Krupp for experimental suggestions. This work was supported by the Deutsche Forschungsgemeinschaft and by the Fond der Chemischen Industrie Deutschlands.

The heptad repeat in the largest subunit of RNA polymerase II binds by intercalating into DNA

Masashi Suzuki*

Laboratory of Neurochemistry, Division of Molecular Physiology,
National Institute for Physiological Sciences, Okazaki 444, Japan

A TANDEM repeat of the sequence Ser-Pro-Thr-Ser-Pro-Ser-Tyr has been found at the C terminus in the largest subunit of RNA polymerase II (refs 1–5) with, for example, 26 units in yeast² and 52 in mammals^{3,5}. By removal of this 'tail', it has been shown that 11–23 units are necessary for the normal functioning of the polymerase^{4,5}. The functional role of the repeat is however, unclear, although it has been proposed that it binds to transcription factors⁶. As discussed in an earlier paper⁷, the repeat unit contains two Ser-Pro sequences which seem to be related to a DNA-binding unit found in histones, Ser-Pro-Lys-Lys⁸, and to the Ser-Pro-X-X motif which is often found in gene regulatory proteins and which, it has been proposed, is also a DNA-binding unit⁷. Here, I show that the repeat does indeed bind DNA and present evidence that it does so by the intercalation of tyrosine residues. These experiments involved synthetic peptides containing one or two repeat units. As the sequence Ser-Pro-X-X (where X represents any amino acid) has a strong tendency to assume a special β -turn⁷, a model of the unit composed of two such β -turns was made and compared

with the structure of the drug Triostin A which is known to intercalate into DNA^{9,10}. Two tyrosine side chains of the repeat overlap well with two quinoxaline rings of the drug and therefore, the model can provide a good explanation of the experimental results.

To test whether the repeating units have the potential to bind DNA, I examined the effect of a synthetic peptide, ⁰Tyr-¹Ser-²Pro-³Thr-⁴Ser-⁵Pro-⁶Ser-⁷Tyr (YY1), with an extra tyrosine residue added to the repeat unit, on the sedimentation constant of the supercoiled replicative form of M13mp18 plasmid DNA. On addition of the peptide, the sedimentation constant decreased to a minimum before returning to its original level (Fig. 1).

As circular DNA produced in *Escherichia coli* is supercoiled in a right-handed manner and as intercalation introduces left-handed superhelicity into DNA, the effect of increasing amounts of an intercalator is first to relax the right handed compact circular DNA until it forms an open circular shape, with a smaller sedimentation constant. As further intercalating molecules are bound, the DNA becomes compact again, assuming a left-handed superhelix with a sedimentation constant

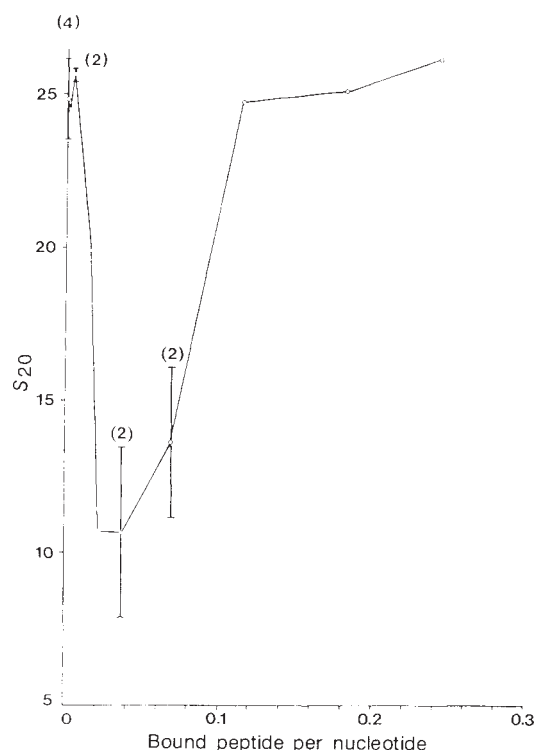


FIG. 1 Effect of the peptide on the sedimentation of the supercoiled circular DNA. The sedimentation constant of the replicative form of the plasmid, M13mp18, at 20 °C (S_{20}) is plotted against the ratio of peptide bound per nucleotide. The number of repetitions of each measurement is shown in parentheses; otherwise the data show the result of one experiment. On addition of the peptide, the S_{20} decreases to a minimum before increasing again to the initial level. This type of curve is characteristic of intercalation into DNA. The binding ratio at the bottom of the curve, 0.03, is very close to that of bifunctional intercalators, Triostin A and its homologues^{13–16}, about 0.028–0.029 and about half that of monofunctional intercalators¹². METHODS. The peptide and the plasmid DNA were purchased from Toray Research Center (Tokyo) and Toyobo Co. (Osaka), respectively. The measurement was carried out at 20 °C with 2.24×10^{-5} M nucleotides and $1.44 \times 7.29 \times 10^{-4}$ M peptide, in 10 mM sodium phosphate buffer (pH 7.0) by using an analytical ultracentrifuge (Hitachi 282). The amount of the peptide binding to DNA was determined by the absorbance of tyrosine moving with DNA at the time of centrifugation. The purchased closed circular DNA was pure and was not contaminated by a nicked circular component.

* Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

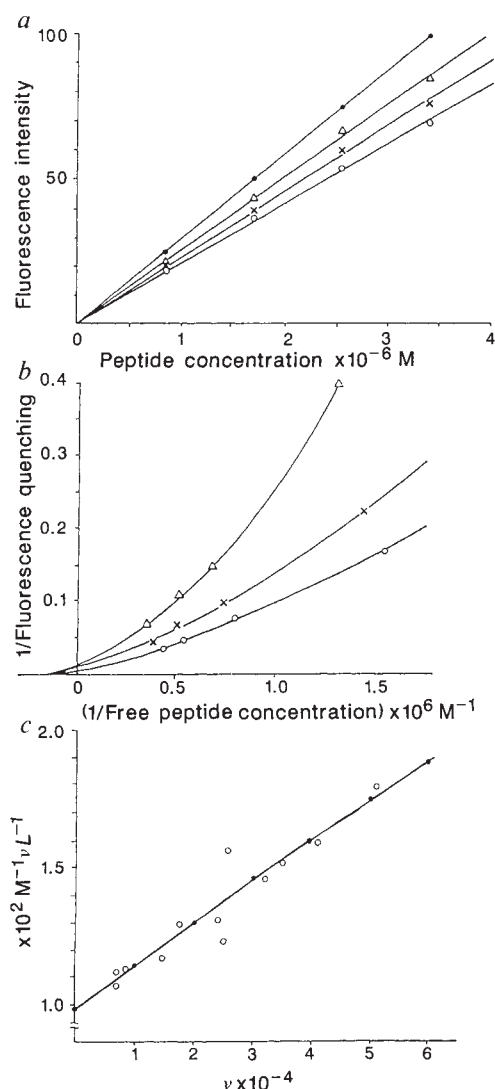


FIG. 2 Fluorescence quenching of tyrosine residues of the peptide, YYY2 by binding to DNA. The fluorescence of tyrosine of YYY2 measured in the absence and presence of linear DNA is shown in a. The fluorescence quenching is due to DNA binding by the peptide. A Lineweaver-Burk plot (b) of $1/C$ against $1/L$, where C and L are the concentrations of peptide in units of moles binding and not binding to DNA respectively, shows highly positive cooperative binding. A Scatchard plot, ν/L against ν , where ν is 'DNA binding density' of the peptide in units of moles of bound peptide per mole of total binding sites, is shown in c. The solid line is calculated (using equation 15, ref. 20) with K , ω , and n equal to 97 M^{-1} , 900 and 6 respectively, where K is the binding constant to the isolated site, ω is the cooperative parameter, and n the number of base pairs covered by one bound peptide. These parameters cannot be estimated uniquely, but the values of K , ω and n must be approximately 100, 1,000 and <16 , respectively. Data for experiments in the absence ($\bullet$) and in the presence of DNA at concentrations of base pairs of $9.91 \times 10^{-4} \text{ M}$ (Δ), $1.98 \times 10^{-3} \text{ M}$ ($\times$), and $2.97 \times 10^{-3} \text{ M}$ ($\circ$) are shown.

METHODS. The fluorescence of tyrosine was measured at different concentrations of the peptide in the range $8.45 \times 10^{-5} \text{ M}$ – $3.38 \times 10^{-6} \text{ M}$, in the absence and presence of salmon testis DNA (Sigma), $9.91 \times 10^{-4} \text{ M}$, $1.98 \times 10^{-3} \text{ M}$, or $2.97 \times 10^{-3} \text{ M}$ (concentration of base pairs), in 10 mM sodium phosphate buffer (pH 7.0). The peptide solution was excited at $273 \pm 2.5 \text{ nm}$ and the fluorescence at $305 \pm 2.5 \text{ nm}$ was measured using a fluorophotometer (Perkin-Elmer LS-5B). As the length of the fully extended 15-mer is $<57 \text{ \AA}$ (that is, $3.8 \text{ \AA} \times 15$) it must cover fewer than 16 base pairs. The intercept on the ν/L axis in c gives a value of K of $\sim 100 \text{ M}^{-1}$. As the maximum slope in the Scatchard plot is equal to $K(2\omega - 2n - 1)$ (ref. 19), the value of $K(2\omega - 2n - 1)$ must be $\sim 1.46 \times 10^5 \text{ M}^{-1}$, the slope of the plot shown in c, which is close to linear in this range. As n is much smaller than ω in this case, the slope is essentially equal to $2K\omega$ and, therefore, ω must be $\sim 1,000$. The fit of the curve is relatively insensitive to a particular combination of parameters, but the ranges of the individual parameters are limited.

similar to that of the initial right-handed superhelix^{11,12}. This kind of behaviour is shown in Fig. 1.

According to Waring¹², at the trough of the sedimentation curve, where DNA has an open shape, the ratio of bound molecule to nucleotide is close to 0.06 for almost all monofunctional intercalators, whereas for bifunctional intercalators such as Triostin A, the ratio is 0.028–0.029 (refs 13–16), which is half that for monofunctional intercalators. The ratio observed for YY1 is close to 0.03 (Fig. 1), almost the same as that for bifunctional intercalators and therefore, YY1 is assumed to behave as a bifunctional intercalator. As the only potential intercalation sites in YY1 are the two aromatic tyrosine rings and as intercalation of tyrosine into DNA has been reported¹⁷, it seems reasonable to expect intercalation by the tyrosine residues of the repeat.

To determine the DNA-binding characteristics of the repeat in more detail, the effect of binding DNA on the fluorescence of tyrosine was examined using linear DNA and a synthetic peptide composed of two repeating units and one additional Tyr, Tyr-Ser-Pro-Thr-Ser-Pro-Ser-Tyr-Ser-Pro-Thr-Ser-Pro-Ser-Tyr (YYY2). When DNA was added to a solution of this peptide, the fluorescence of tyrosine was quenched (Fig. 2a). This is also consistent with the idea of intercalation, because the fluorescence of tyrosine is expected to decrease to a value close to zero when it intercalates into DNA¹⁸.

A Lineweaver-Burk plot (Fig. 2b) of the data shows lines which are not linear but curve downwards, as is expected for positive cooperative binding. Such cooperativity might be induced by a change in the local DNA structure produced by the intercalation of one peptide, which makes it easier for a second peptide to intercalate nearby.

These measurements of DNA binding by YYY2 were analysed according to the cooperative DNA-binding theory of McGhee and von Hippel¹⁹, where a cooperativity parameter, ω , is introduced such that the binding constants for isolated, singly and doubly contiguous sites are K , $K\omega$ and $K\omega^2$, respectively. The Scatchard plot is shown in Fig. 2c.

The results were calibrated for the changes in fluorescence produced by absorption by DNA (a decrease) or by Raman scattering by water (an increase), by measuring their effects on the fluorescence of free tyrosine. The peptide YYY2 was used for the fluorescence measurements rather than the shorter YY1 peptide, simply because it has a higher binding constant (personal observation). If free tyrosine were weakly intercalated with DNA, then the measured binding constant would be an underestimate, especially when the binding constant of YYY2 is small. This might produce an overestimate of the cooperativity in DNA binding.

When I tried to use YYY2 for the sedimentation studies on circular DNA and repeated the centrifugation experiments, I

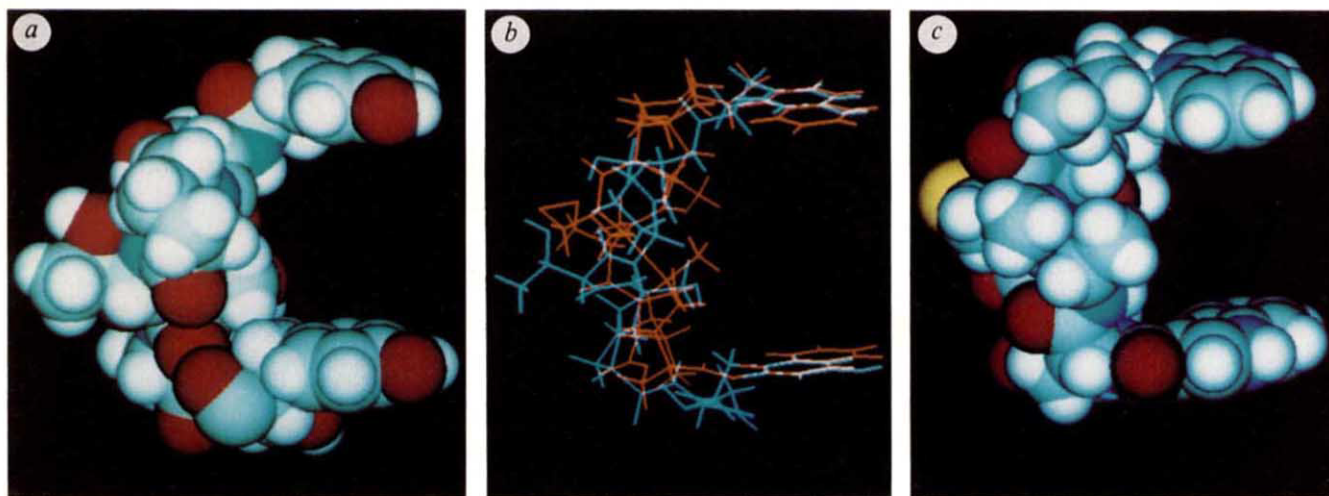


FIG. 3 Comparison of the proposed structure of the repeat unit with the structure of Triostin A. The proposed structure of YY1 (a) is compared with the structure of Triostin A (c) which intercalates into DNA, sandwiching two G-C base pairs²⁷. The two molecules are shown with the atomic colour code: blue for N, light green for C, yellow for S and red for O. The proposed structure of the peptide (blue in b) can be superimposed onto the structure of Triostin A (red in c) as a whole, with the distance between the two tyrosine side chains, 10.2 Å, being the same as that of two quinoxaline rings. The experimental result can, therefore, be explained by the bifunctional

intercalation of the two tyrosine residues in the YY1 model into DNA. Note that Triostin A, and probably YY1, bind to a minor groove of DNA, as well as another Ser-Pro-containing peptide, Ser-Pro-Lys-Lys binds to the minor groove^{8,28}.

METHODS. Two β -turn structures were made, essentially with the coordinates used previously⁸. The ϕ angle of ⁴Ser was rotated. The model shown here was built with the dihedral angles (ϕ, ψ) being ($-150.9^\circ, 103.7^\circ$), ($-163.7^\circ, 133.8^\circ$), ($-60.1^\circ, -24.4^\circ$), ($-90.0^\circ, 22.8^\circ$), ($-55.1^\circ, 132.3^\circ$), ($-60.0^\circ, -10.2^\circ$), ($-113.7^\circ, 23.1^\circ$) and ($-150.9^\circ, 103.7^\circ$).

observed two completely different sedimentation constants for the same specimen. Sometimes the value changed according to the concentration of the peptide, just as with YY1, but in other cases it was the same as the sedimentation constant without the peptide. This sort of erratic behaviour would be expected for a trifunctional intercalator such as YYY2. Unlike a monofunctional intercalator, its awkward geometry might lead to very unstable structures, with the peptides being released from DNA in a cooperative manner. Note that complications were also observed for measurement of sedimentation constants when other synthetic tris-intercalators were used (M. Waring *et al.*, personal communication; and ref. 21). Linear DNA was used for the fluorescence measurements to avoid these complications.

As the mode of intercalation of YY1 seems to resemble that of Triostin A, it was interesting to see whether they have similar structures. I stabilized the special β -turn often assumed by Ser-Pro-XX with a second hydrogen bond between the oxygen atom of the serine side chain and the amide of the third residue, in addition to the hydrogen bond between CO of the first residue and NH of the fourth residue generally found in β -turns^{7,8,22}. The structure of such a β -turn is characterized by five dihedral angles: the ψ angle of serine and two sets of the dihedral angles, ϕ and ψ , of proline and the third residue. When two such β -turns were assumed by the ¹Ser-²Pro-³Thr-⁴Ser and ⁴Ser-⁵Pro-⁶Ser-⁷Tyr sequences of YY1, there is only one degree of freedom, that of ϕ of ⁴Ser, to change the connection between two β -turns.

Using computer graphics, the ϕ angle of ⁴Ser was rotated and, with the dihedral angles given in the Fig. 3 legend, a good correspondence between the two tyrosine side chains and quinoxaline rings was found (Fig. 3b). In this model, the distances between the two C β 's and O's of the two tyrosine residues are 11.1 Å and 10.2 Å, values close to those observed when the two quinoxaline rings of Triostin A are co-crystallized with short DNA^{9,10} (Fig. 3c).

These data show that the two synthetic peptides bind to DNA. The characteristics of this binding are wholly consistent with intercalation being the dominant mode of interaction. It is unknown at present whether the repeat also binds to RNA. The

arrangement of tyrosine residues in the repeat is different from that in the consensus sequence reported for RNA binding²³; thus, if the repeat binds to RNA, it does so in a different way.

We do not know whether the structure of an isolated repeat unit corresponds exactly to that of the same amino-acid residues incorporated into the folded domain of polymerase II or whether the C terminus interacts significantly with DNA *in vivo*. However, the Ser-Pro-X-X structural unit is thought to be fairly stable^{7,8} and the heptad unit which is known to bind DNA *in vitro*, is very likely to be able to bind DNA *in vivo* as well.

As intercalation changes the local structure of DNA, the repeat may bind to the transcription region of the DNA and have an important role in changing the DNA structure, perhaps promoting DNA-RNA hybridization, as does another DNA-binding protein, G5BP, which binds to and destabilizes double-stranded DNA by intercalation of its tyrosine residues²⁴. As the DNA-binding constant of the unit is lower than that of G5BP, the repeat may slide along, unzipping the double helix.

Another possible role for the DNA-binding property of the heptad repeat is to anchor RNA polymerase to the promoter region of the DNA, thus becoming involved in regulating transcription. This idea is consistent with the suggestion that the phosphorylation of the heptad repeat might be necessary for the elongation of RNA chains²⁶, because, after the initiation of transcription, phosphorylation, which introduces as negative charges, might weaken the DNA-binding properties of the repeat. The heptad repeat can be multifunctional and the results reported here are compatible with the suggestion^{6,26} that the repeat interacts with transcription factor as well as with DNA. The threonine residue in my model (Fig. 3) is directed outwards from the DNA and seems capable of such an interaction. □

Received 8 November 1989; accepted 18 January 1990.

1. Corden, J. L., Cadena, D. L., Ahearn, J. M. & Dahmus, M. E. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7934-7938 (1985).
2. Allison, L. A., Moyle, M., Shales, M. & Ingles, C. J. *Cell* **42**, 599-610 (1985).
3. Ahearn, J. M., Bartolomei, M. S., West, M. L., Cisek, L. J. & Corden, J. L. *J. biol. Chem.* **262**, 10695-10705 (1986).

4. Zehring, W. A., Lee, J. M., Weeks, J. R., Jorke, R. S. & Greenleaf, A. L. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3698-3702 (1988).
5. Allison, L. A., Wong, J.K.-C., Fitzpatrick, V. D., Moyle, M. & Ingles, C. J. *molec. Cell Biol.* **8**, 321-329 (1988).
6. Sigler, P. B. *Nature* **333**, 210-212 (1988).
7. Suzuki, M. *J. molec. Biol.* **207**, 61-84 (1989).
8. Suzuki, M. *EMBO J.* **8**, 797-804 (1989).
9. Wang, A.H.-J. et al. *Science* **225**, 1115-1121 (1984).
10. Quigley, G. J. et al. *Science* **232**, 1255-1258 (1986).
11. Crawford, L. V. & Waring, M. J. *J. molec. Biol.* **25**, 23-30 (1967).
12. Waring, M. J. *J. molec. Biol.* **54**, 247-279 (1970).
13. Waring, M. J. & Wakelin, L. P. G. *Nature* **252**, 653-657 (1974).
14. Wakelin, L. P. G. & Waring, M. J. *Biochem. J.* **150**, 721-740 (1976).
15. Lee, J. S. & Waring, M. J. *Biochem. J.* **173**, 129-144 (1978).
16. Lee, J. S. & Waring, M. J. *Biochem. J.* **173**, 115-128 (1978).
17. Hélène, C., Monttenay-Garestier, T., & Dimicoli, J.-L. *Biochim. biophys. Acta* **154**, 349-365 (1970).
18. Hélène, C. *Nature New Biol.* **234**, 120-121 (1971).
19. McGhee, J. D. & von Hippel, P. J. *J. molec. Biol.* **86**, 469-489 (1974).
20. McGhee, J. D. & von Hippel, P. J. *J. molec. Biol.* **103**, 679 (1976).
21. Laugåa, P., Markovitz, J., Delbarre, A., LePec, J.-B. & Roques, B. P. *Proc. natn. Acad. Sci. U.S.A.* **24**, 5567-5575 (1985).
22. Wilmot, C. M. & Thornton, J. M. *J. molec. Biol.* **203**, 221-232 (1988).
23. Dreyfuss, G., Swanson, M. S. & Rinol-Roma, S. *Trends Biochem. Sci.* **13**, 86-91 (1988).
24. Alberts, B., Frey, L., & Delius, H. J. *J. molec. Biol.* **68**, 139-152 (1972).
25. Bartholomew, B., Dahmus, M. E. & Meares, C. F. *J. biol. Chem.* **261**, 14226-14231 (1986).
26. Allison, L. A. & Ingles, C. J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2749-2798 (1989).
27. Wang, A.H.-J., Ughetto, G., Quigley, G. J. & Rich, A. J. *biomolec. Struct. Dynamics* **4**, 319-342 (1972).
28. Churchill, M. E. A. & Suzuki, M. *EMBO J.* **8**, 4189-4195 (1990).

ACKNOWLEDGEMENTS. I thank A. Klug and A. Travers for comments and G. Jacobs and Y. Iga (System Co.) for advice on the computer graphics study. I also thank M. Seto and H. Hattori (National Institute for Basic Biology) for technical assistance. This work was supported by the Ministry of Education, Science and Culture, Japan.

Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity

Jean-Pierre Perreault*, Taifeng Wu†‡, Benoit Cousineau*, Kelvin K. Ogilvie†‡ & Robert Cedergren*§

*Département de biochimie, Université de Montréal, Québec H3C 3J7, Canada

†Department of Chemistry, McGill University, Montréal, Québec H3A 2K6, Canada

THE RNA of viroids and virusoids in plants, and the RNA transcripts of some tandemly repeated DNA sequences in the newt, can undergo self-catalysed cleavage to generate RNA with 5'-OH and 2',3'-cyclic-phosphate termini¹⁻⁴. These catalytic RNAs, or ribozymes, form a stem-loop secondary structure called a 'hammerhead' in which the catalytic (ribozyme) and substrate sequences are brought close together (Fig. 1a). Catalytically active mimics of hammerhead ribozymes can be readily made using oligoribonucleotides⁵⁻⁷. Consequently, hammerhead analogues in which certain ribonucleotides are replaced by different ones have been constructed both to identify consensus residues required for cleavage activity and to determine the details of the cleavage mechanism⁸⁻¹⁰. But these ribonucleotide-replacements tend to alter the conformation of the hammerhead by changing hydrogen-bonding and stacking potential at the position of substitution. We have now constructed structurally less-disrupted hammerhead analogues in which deoxyribonucleotides, which lack 2'-OH groups, are substituted for ribonucleotides. These mixed RNA-DNA polymers¹¹ were synthesized using a strategy for the chemical synthesis of RNA that is compatible with DNA synthesis¹². Analysis of the cleavage products of several of these hammerhead analogues confirms the involvement in the reaction of the 2'-OH adjacent to the cleavage site in the substrate, and demonstrates that some 2'-OH groups in the catalytic region strongly affect

activity. The results also indicate that the three-dimensional structure producing nucleic acid-type catalysis is not restricted to RNA.

First, we demonstrated the fidelity of the RNA chemical synthesis method^{12,13} by synthesizing and characterizing the all-RNA ribozyme and substrate shown in Fig. 1b. Incubation of the 5' ³²P-labelled substrate with the ribozyme resulted in cleavage to the expected octanucleotide (Fig. 2a). To further characterize the products, we cleaved an unlabelled substrate, labelled the 3' termini of the products with [³²P]pCp in the presence of RNA ligase, and analysed them by PAGE (Fig. 2a, lane 4). This mixture gave three labelled bands: (1) a slow-moving band corresponding to the labelled ribozyme; (2) a band of intermediate speed comprising the labelled six-residue 3' cleavage fragment; and (3) a band consisting of unreacted [³²P]pCp. This experiment also confirmed that the 5' cleavage fragment possesses a 2',3'-cyclic phosphate, as it was not labelled during incubation. Finally, 5' and 3' end-group analyses of the substrate and the two cleavage products confirmed the site of hydrolysis and the structure of the products (data not shown).

For the reaction with the chemically synthesized substrates, the Michaelis constant $K_m = 0.92 \mu\text{M}$, and the catalytic constant $k_{cat} = 2.6 \text{ min}^{-1}$. This compares with a K_m of $0.62 \mu\text{M}$ and a k_{cat} of 0.48 min^{-1} for the 19-residue ribozyme/24-residue substrate made by *in vitro* transcription⁵. The 19-residue ribozyme/24-residue substrate of the Uhlenbeck model⁵ prepared by chemical synthesis showed activity levels comparable to those of transcribed RNA. Finally, 'ribozymes' made entirely from deoxyribonucleotides (of both the Haseloff-Gerlach model⁷ and the Uhlenbeck model⁵) had no activity. These results demonstrate that the chemically synthesized ribozyme is virtually equivalent to RNA made by *in vitro* transcription, and support the use of mixed DNA-RNA polymers to evaluate the role of ribozyme and substrate 2'-OH groups in catalysis.

RNA-catalysed cleavage reactions can be classified into two groups according to how the phosphodiester bond is broken¹⁴.

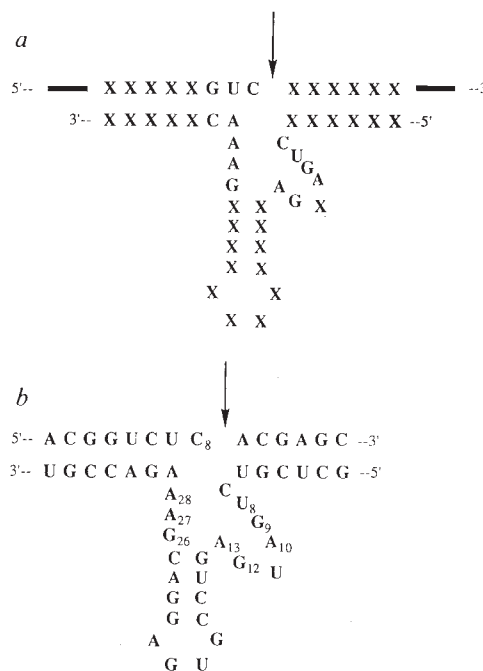


FIG. 1 a, Consensus structure of the hammerhead catalytic RNA (self-cleaving-type ribozyme) in the model of Haseloff-Gerlach⁷. b, RNA structure produced by chemical synthesis. The single-stranded consensus nucleotides that were substituted with deoxyribonucleotides are numbered. Note that the G6:C30 base pair present in the consensus structure (a) was changed to C6:G30 in the synthetic molecules; this substitution does not affect catalytic efficiency⁹.

† Present addresses: Acadia University, Wolfville, Nova Scotia B0P 1X0, Canada (K.K.O.); The Salk Institute for Biological Studies, San Diego, California 92138, USA (T.W.).

§ To whom correspondence should be addressed.

The cleavages catalysed by self-splicing (introns of group I) RNA¹⁵ and ribonuclease P¹⁶ produce 5'-P/3'-OH fragments or intermediates. Self-cleaving hammerhead RNA produces 5'-OH/2,3-cyclic-phosphate products, suggestive of the RNA cleavage mechanism in which phosphodiester bond hydrolysis is initiated by nucleophilic attack of the adjacent 2'-OH (or an activated derivative of it). Mg²⁺, required for hammerhead cleavage, could increase either the nucleophilicity of the 2'-OH or the electrophilic nature of the phosphate, as does Pb²⁺ in Pb²⁺-catalysed RNA hydrolysis¹⁷. In both cases, however, an adjacent 2'-OH would be an essential feature of the cleavage site.

We tested whether such a 2'-OH group is required for cleavage by preparing an oligonucleotide substrate having the sequence of the substrate shown in Fig. 1b, except that the ribocytidine at position 8 (from the 5' terminus) was replaced by 2'-deoxyribocytidine (the dC8 analogue). Under conditions that promote virtually quantitative cleavage of the all-RNA substrate, the ³²P-labelled dC8 analogue was not cleaved (Fig. 2b). These results indicate that the 2'-OH next to the cleavage site is directly involved in the ribozyme-catalysed cleavage. The lack of cleavage of a substrate containing a 2'-O-methylnucleoside also supports this model¹⁸.

Catalytic-region 2'-OH groups are thought to be involved in the cleavage reaction, because no DNA molecule has yet been demonstrated to have catalytic activity. To assess the importance of these 2'-OH groups, we substituted up to a total of eight consensus ribonucleotides with deoxyribonucleotides in three single-stranded domains (Fig. 1b; positions 8–10, 12–13 and 26–28) of the catalytic region. Two mixed oligomers, one with deoxyribonucleotides substituted at all of these eight positions (the 8D-ribozyme), and the other with deoxyribonucleotides substituted at positions 12–13 and 26–28 (the 5D-ribozyme), were synthesized and gel-purified. Incubation of the mixed ribozymes with the 14-residue RNA substrate revealed that the 5D analogue has an activity between that of the all-RNA ribozyme and the 8D analogue (Fig. 3a). We therefore conclude that one or all of the three 2'-OH groups present in the 5D

TABLE 1 Kinetic parameters for ribozyme catalysis

Ribozyme	$V_{\max}$ ($\mu\text{mol min}^{-1}$)	K_m (μM)	Relative $V_{\max}/K_m$
All-RNA	2.00	0.92	1.0000
5D-ribozyme	0.086	0.92	0.0430
8D-ribozyme	0.010	0.96	0.0048
6DA-ribozyme	0.066	0.97	0.0310
6DG-ribozyme	0.011	0.90	0.0056

The reactions were performed as described in Fig. 2 legend. Four substrate concentrations were used, varying between 0.233 and 5.84 μM , whereas the ribozyme concentration was 0.048 μM .

analogue and absent in the 8D analogue (nucleotides 8, 9 and 10) are involved in catalysis.

To further elucidate this hydroxyl group requirement, we prepared two additional mixed polymers: the 6DA-ribozyme (deoxyribonucleotide substitution at positions 12–13, 26–28 and dA10) and the 6DG-ribozyme (deoxyribonucleotide substitution at positions 12–13, 26–28 and dG9). The hydroxyl of rG9 is implicated in ribozyme efficiency, because 6DA had nearly as much activity as 5D, whereas 6DG had only the activity of the 8D analogue (Fig. 3a).

We determined the kinetics of catalysis for all derivatives. Figure 3b presents, as an example, the time course of the reaction catalysed by the 5D-ribozyme. Kinetic parameters, including the apparent K_m and the reaction velocity at infinite substrate concentration ($V_{\max}$), for each ribozyme analogue are given in Table 1. The values were obtained for substrate concentrations up to 125 times the concentration of the ribozyme and greater than six times the K_m . K_m values for all the ribozymes were very similar. $V_{\max}$ varies up to 200-fold between the $V_{\max}$ of the all-RNA ribozyme and the $V_{\max}$ of D8 and 6DG. The kinetics of 6DA approaches that of 5D, confirming the specific effect of the 2'-OH of G9 on ribozyme activity.

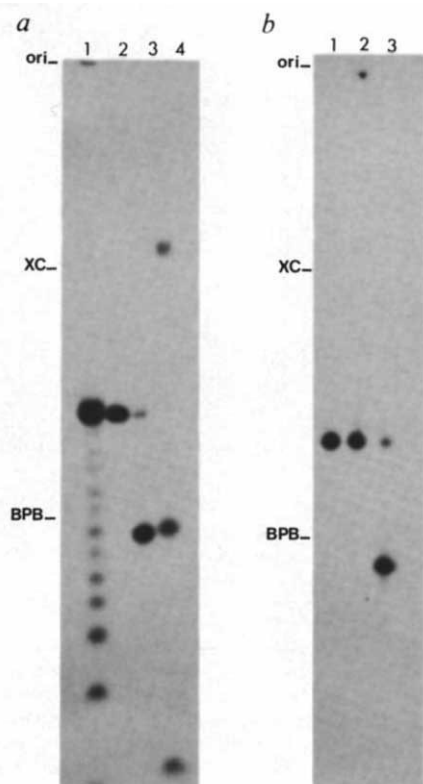


FIG. 2 a, Lane 1, RNA ladder of 5'-[³²P]labelled 14-residue substrate produced by partial base hydrolysis; lane 2, unreacted 5'-[³²P]labelled 14-residue substrate; lane 3, 5'-[³²P]labelled substrate incubated with the ribozyme under the conditions described below; lane 4, unlabelled substrate was reacted with ribozyme, and products 3' end-labelled with [³²P]pCp. The top band corresponds to 3'-labelled ribozyme; the middle band corresponds to labelled 3' cleavage fragment, and migrates more slowly than the eight-residue product of ribozyme hydrolysis in other lanes because it lacks a 5' terminal phosphate and contains an additional pCp. The fastest-moving band is that of unreacted [³²P]pCp. b, Activity of the dC8 substrate analogue. Lane 1, 5'-[³²P]ACAGGUCUCACGAC-3' all-RNA substrate treated as described below, but with no added ribozyme; lane 2, reaction with 5'-[³²P]ACGGUCUGCAGCAGC-3' (dC8 analogue) and ribozyme; lane 3, labelled all-RNA substrate with added ribozyme. Abbreviations: ori, origin; xc, xylene cyanol; BPB, bromo-phenol blue.

METHODS. All oligonucleotides synthesized on automatic oligonucleotide synthesizers as described elsewhere^{11–13}. Deoxyribonucleotide intermediates were the cyanoethylphosphoramidites and ribonucleotide intermediates, the 2'-*t*-butyldimethylsilylated nucleoside 3'-methylphosphoramidites. Oligomers (that is, deoxyribonucleotides, ribonucleotides, and mixed deoxyribonucleotides and ribonucleotides) were gel-purified, and the oligoribonucleotides sequenced using rapid-RNA gel-sequencing techniques. All radioactive oligonucleotides were 5'-[³²P]labelled using polynucleotide kinase and [³²P]ATP. Labelling of 3' termini was performed by T4 RNA ligase and [³²P]pCp. Reaction conditions for ribozyme cleavages: substrate (0.233 μM) and ribozyme (0.044 μM) were heated to 65 °C for 1 min in 75 mM Tris-HCl buffer, pH 7.5. After rapid cooling, MgCl₂ was added to 10 mM, and the mixture was incubated for 2 h at 37 °C. The reaction was stopped by addition of formamide-dye, and the resulting mixture was analysed by 20% PAGE in 50 mM Tris/boric acid containing 1 mM EDTA and 7 M urea, pH 8.3. The positions of the dye markers are indicated.

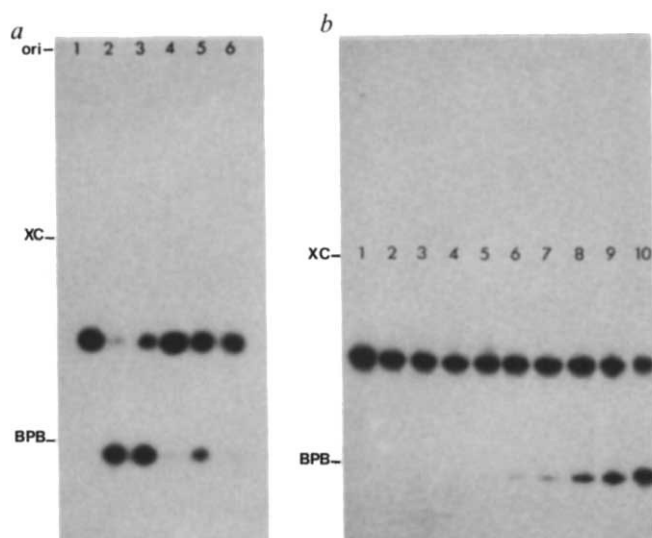


FIG. 3 *a*, Cleavage of all-RNA 5'-[32 P] substrate with various deoxyribonucleotide-containing analogues of the ribozyme. Lane 1, substrate incubated as described in Fig. 2 without added ribozyme; lane 2, reaction of labelled substrate with all-RNA ribozyme; lane 3, reaction of labelled substrate with 5D-ribozyme; lane 4, reaction of labelled substrate with 8D-ribozyme; lane 5, reaction of labelled substrate with 6DA-ribozyme; lane 6, reaction with 6DG-ribozyme. *b*, Time course of cleavage reaction using 5D-ribozyme. Lanes 1–10: 0, 0.5, 1, 2, 5, 10, 20, 60, 120 and 240 min, respectively, using the conditions described in Fig. 2 with a substrate-ribozyme ratio of 125.

Interpreting the different ribozyme activities in terms of the structure of the polymers is complicated by the fact that the reduced activities of the deoxyribonucleotide-containing molecules could result from conformational differences among the analogues, a direct involvement of 2'-OH groups in catalysis, or a nonspecific 'relaxation' of the structure. The difference between the activities of the 6DA and 6DG analogues, however, argues against a general relaxation and favours a specific-conformational or direct-catalytic effect. Indeed, there are conformational differences between double-stranded polyribonucleotides and polydeoxyribonucleotides. The carbohydrate moiety of deoxyribonucleotides adopts the 2'-endo conformation, which is thought to direct the formation of the DNA B-type helix; ribonucleotides, tending to adopt the 3'-endo conformation, generally form A-type helices^{19,20}. But, high-resolution NMR data²¹ show that 3'-endo and 2'-endo strands can coexist in heteroduplexes of RNA and DNA strands, indicating that only minor conformational differences would be expected in helical regions on deoxyribonucleotide substitution. Structural differences between deoxyribonucleotides and ribonucleotides in single-stranded regions of the ribozyme are much less likely²², because the conformation of both would be sequence-dependent, that is, related to the base-pairing or stacking of the nucleotides^{19,23,24}. The almost identical K_m values of the native ribozyme and the mixed ribozymes argue against major conformational changes in these analogues; such changes would alter the double-helical substrate-binding site and therefore the K_m . But more-restrained conformational effects in the single-stranded region of the ribozyme cannot be ruled out.

In catalysis, ribozyme 2'-OH groups could be involved in

binding Mg^{2+} (ref. 25). Although there is no evidence that binding of Mg^{2+} to 2'-OH is direct, it could occur through solvent bridges²⁶. Based on the coordination of Mg^{2+} , up to three 2'-OH groups could be among the functional groups involved in binding. The absence of any of these specific 2'-OH groups would necessarily reduce Mg^{2+} binding and thereby ribozyme activity.

Although we cannot yet distinguish between conformational or catalytic involvement in cleavage, it would be useful to determine which 2'-OH is involved so that the structural basis of catalysis may be modelled. Our results demonstrate that the 2'-OH of rG9 is one of the critical hydroxyl groups. In support of this, a recent model of the ribozyme, calculated from an energy-minimization routine, places G9 close to Mg^{2+} (ref. 27). The higher activity of the all-RNA ribozyme indicates that other 2'-OH groups in the other five substituted positions of the 5D-ribozyme could also be involved in the cleavage reaction.

The discovery of catalytic RNA, and the fact that no nucleic acid other than RNA has been demonstrated to have catalytic properties, has given support to the suggestion that RNA was the first informational macromolecule. But Orgel²⁸ argues that the lack of ribonucleotide synthesis in primordial 'broths' implies that other nucleotide-like polymers could have preceded RNA as the primitive catalyst. Therefore nucleic acid-type catalysis might not be an exclusive property of RNA. Our finding that nucleic-acid polymers containing deoxyribonucleotides can have catalytic properties raises both the question of what fundamental structural requirements are necessary for catalytic activity, and the prospect that other polymers, related to nucleic acids, have catalytic activity. □

Received 2 November 1989; accepted 5 February 1990.

- Buzayan, J. M., Gerlach, W. L. & Bruening, G. B. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8859–8862 (1986).
- Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. *Nucleic Acids Res.* **14**, 3627–3640 (1986).
- Forster, A. C. & Symons, R. H. *Cell* **49**, 211–220 (1987).
- Epstein, L. M. & Gall, J. G. *Cell* **48**, 535–543 (1987).
- Uhlenbeck, O. C. *Nature* **328**, 596–600 (1987).
- Jeffries, A. C. & Symons, R. H. *Nucleic Acids Res.* **17**, 1371–1377 (1989).
- Haseloff, J. & Gerlach, W. L. *Nature* **334**, 585–591 (1988).
- Sampson, J. R., Sullivan, F. X., Behlen, L. S., DiRenzo, A. B. & Uhlenbeck, O. C. *Cold Spring Harb. Symp. quant. Biol.* **52**, 267–275 (1987).
- Koizumi, M., Iwai, S. & Ohtsuka, E. *FEBS Lett.* **228**, 228–230 (1988).
- Sheldon, C. C. & Symons, R. H. *Nucleic Acids Res.* **17**, 5679–5685 (1989).
- Wu, T., Ogilvie, K. K., Perreault, J. P. & Cedergren, R. *J. Am. chem. Soc.* **111**, 8531–8533 (1989).
- Usman, N., Ogilvie, K. K., Jiang, M. Y. & Cedergren, R. *J. Am. chem. Soc.* **109**, 7845–7854 (1987).
- Wu, T., Ogilvie, K. K. & Pon, R. T. *Nucleic Acids Res.* **17**, 3501–3517 (1989).
- Cedergren, R., Lang, B. F. & Gravel, D. *FEBS Lett.* **226**, 63–66 (1987).
- Zaug, A. J., Been, M. D. & Cech, T. *Nature* **324**, 429–433 (1986).
- Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. *Cell* **35**, 849–857 (1983).

- Brown, R. S., Dewan, J. C. & Klug, A. *Biochemistry* **24**, 4785–4801 (1985).
- Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H. & Ohtsuka, E. *Nucleic Acids Res.* **17**, 7059–7070 (1989).
- Dickerson, R. E. *J. molec. Biol.* **166**, 419–441 (1983).
- Dock-Bregeon, A. C. et al. *Nature* **335**, 375–378 (1988).
- Chou, S.-H., Flynn, P. & Reid, B. *Biochemistry* **28**, 2435–2443 (1989).
- Paquette, J., Nicoghossian, K., Qi, G.-R., Beauchemin, N. & Cedergren, R. *Eur. J. Biochem.* (in the press).
- Saenger, W. *Principles of Nucleic Acid Structure*, 331–344 (Springer, New York, 1984).
- van den Hoogen, Y. et al. *Eur. J. Biochem.* **173**, 295–303 (1988).
- Joyce, G. F. *Nature* **338**, 217–224 (1989).
- Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S. & Klug, A. *J. molec. Biol.* **111**, 315–328 (1977).
- Mei, H.-Y., Kaaret, T. W. & Bruce, T. C. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9727–9731 (1989).
- Orgel, L. E. *Cold Spring Harb. Symp. quant. Biol.* **52**, 9–16 (1987).

ACKNOWLEDGEMENTS. We thank Olke Uhlenbeck for helpful discussion, John Bratty for technical assistance and Sylvie Ricard for assistance in preparing the figures. This work was supported by the Medical Research Foundation (R.C.) and the Natural Science and Engineering Research Council (K.K.O.). J.-P.P. was supported by a predoctoral fellowship from the FCAR Québec; R.C. is a fellow of the Canadian Institute of Advanced Research.

Protection against UV-induced pyrimidine dimerization in DNA by triplex formation

Victor I. Lyamichev*, Maxim D. Frank-Kamenetskii* & Valery N. Soyfer†

Department of Molecular Genetics, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210-1292, USA

CYCLOBUTANE and [6-4]-pyrimidine dimers are major photo-products of ultraviolet-irradiated DNA. The yield of these photo-products is dependent on the sequence^{1,2} and structure³ of the DNA. By analysing the photofootprints of fragments produced by cleavage of the DNA chain near [6-4]-pyrimidine dimers⁴, we show here that a homopurine-homopyrimidine insert (with either d(TC)_x or d(C)_n) in plasmid pUC19 is, as expected, a good target for UV-induced pyrimidine-dimer formation. But we find that dimerization is virtually completely suppressed when the pyrimidine oligonucleotides d(TC)_y or d(C)_m are added to DNA carrying d(TC)_x- or d(C)_n-containing inserts, respectively. This effect is dependent on the type of oligonucleotide used and is site-specific. The protection occurs under acidic conditions that favour the formation of intermolecular triplexes between the homopurine-homopyrimidine inserts and homologous oligopyrimidines⁵. We therefore conclude that triplex formation effectively protects the DNA duplex from UV-induced damage (pyrimidine dimerization). This observation makes the photofootprinting assay⁶ a very promising method for studying intermolecular and intramolecular triplexes (H-form DNA) both *in vitro* and *in vivo*.

Figure 1 shows the data for both homopurine-homopyrimidine inserts that we studied. In the absence of oligonucleotides (lanes 1-4 in Fig. 1a; lanes 3 and 6 in Fig. 1b) or in the presence of oligonucleotides at neutral pH (lanes 2 and 5, in Fig. 1b) both homopurine-homopyrimidine inserts were heavily damaged by UV-irradiation. But, there was no dimerization or only very weak modification in the presence of the oligonucleotides under acidic conditions (lanes 5 and 6 in Fig. 1a; lanes 4 and 7 in Fig. 1b). Previous experiments have demonstrated that under these conditions the inserts and the oligonucleotides specifically form intermolecular triplexes with each other⁷. We therefore conclude that formation of intermolecular triplexes virtually prevents the UV-induced damage of duplex DNA, at least the formation of [6-4]-pyrimidine dimers.

To determine the influence of different oligonucleotides, we added d(C)₁₀, d(CT)₅, d(G)₁₀ and d(AG)₅ oligonucleotides to DNA carrying the d(C)₁₈-containing insert (Fig. 2). As expected, at neutral pH under which the formation of triplexes is hindered, there was no protection with either of the oligonucleotides (Fig. 2, lanes 6-10), whereas at pH 4.3, there was strong protection with d(C)₁₀ (lane 2). Neither d(CT)₅ nor d(G)₁₀ protected against pyrimidine dimer formation (lanes 3, 4, 8 and 9 in Fig. 2). Unexpectedly, d(AG)₅ partially protected against dimer formation (lane 5). This can be explained by the formation of a non-canonical triplex with CGG- and protonated CGA⁺-base triads. The nature of the effect of d(AG)₅ needs further investigation.

In the next series of experiments performed with DNA carrying two d(TC)₁₆-containing inserts, we observed protection only after the addition of the d(CT)₅ oligonucleotide and only at low pH (Fig. 3, lane 4). Oligonucleotides d(C)₁₀, d(G)₁₀ and d(AG)₅ had no effect on the yield of pyrimidine dimers (see Fig. 3, lanes 3, 5, 6, 8, 10, and 11). These experiments clearly show that

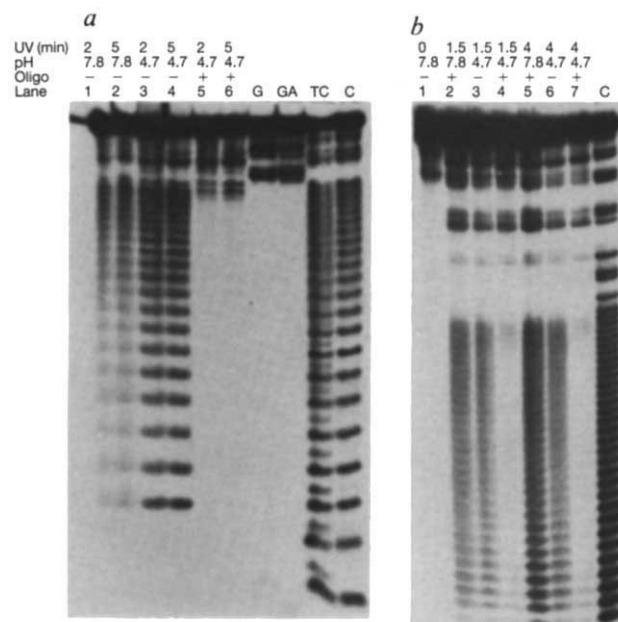


FIG. 1 Detection of pyrimidine dimers in the DNA strand carrying the polypyrimidine d(TC)₂₁- and d(C)₃₁-containing inserts. The brackets define the insert regions. The 3' ends of the fragments are at the bottom. a, Lanes 1-4, control experiments without an oligonucleotide (-); lanes 5 and 6, experiments with d(CT)₅ oligonucleotide (+). Irradiation was performed in a buffer (0.1 M NaCl; 10 mM Tris-HCl, 1 mM EDTA) at either pH 7.8 or with sodium acetate, pH 4.7, for either 2 min or 5 min, as indicated. Lanes G, A, T, C, standard Maxam-Gilbert ladders. b, Lanes 1, 3 and 6, control experiments without oligonucleotide (-); lanes 2, 4, 5 and 7 experiments with d(C)₁₂₋₁₈ (+). Irradiation was performed at either pH 7.8 or pH 4.7 for 0, 1.5 or 4 min, as indicated. Lane C, standard Maxam-Gilbert ladder for C residues. Oligo, oligonucleotide.

METHODS. The plasmid carrying d(TC)₂₁- or d(C)₃₁-containing inserts were prepared as described^{7,8}. The d(TC)₂₁-containing insert was incorporated into *Bam*HI-*Eco*RI site of the pUC19 plasmid. The d(C)₃₁-containing insert was inserted into the *Pst*I site of pUC19 plasmid. Plasmid DNA (1 µg) was first linearized either with *Eco*RI (for d(TC)₂₁) or *Hind*III (for d(C)₃₁) and ³²P-end-labelled with the Klenow fragment of DNA polymerase I. The samples were digested with either *Hind*III (for d(TC)₂₁) or *Eco*RI (for d(C)₃₁), and the preparations were dissolved in the buffer (see above). About 0.2 µg unlabelled d(TC)₅ (for d(TC)₂₁) or unlabelled d(C)₁₂₋₁₈ (for d(C)₃₁) was added, and the sample was irradiated by a germicidal lamp (15 W, 254 nm) for either 2 or 5 min. After irradiation, the small uniquely labelled fragments (5'-AGCTTGCATGCTGCGAGGTCGACTCTAGAGGATCC(TC)₂₁GAA-3' or 5'-AATTCGAGCTCGGTACCGGGGATCCTCTAGAGTCGACCTGCA(C)₃₁TGCAGGCATGCAAGC-3') were isolated and treated with 1M piperidine (90 °C, 30 min) and run in a denaturing gel.

protection occurs predominantly under conditions facilitating triplex formation between the homopurine-homopyrimidine duplex and homologous pyrimidine oligonucleotides.

For DNA surrounding the homopurine-homopyrimidine inserts, the photofootprints were the same for samples with and without oligonucleotides (see upper parts of the radioautographs in Fig. 1, and upper and lower parts in Figs 2 and 3). Protection is therefore highly site-specific. End-effects were also evident for the d(C)₃₁ insert: both ends of the insert were weakly damaged by the UV irradiation (Fig. 1b).

The sequence-specificity of cleavage of duplex DNA generally agreed with the characteristic pattern of cleavage near [6-4]-pyrimidine dimers by piperidine⁴. In accordance with the pattern, we observed cleavage at the C position in TC and CC sites.

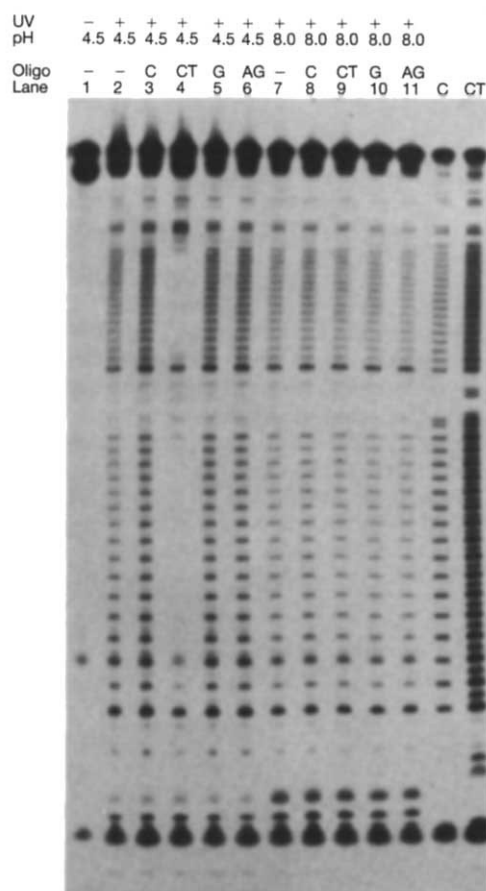
It has been suggested that triplex stability strongly depends on pH (ref. 5). This is confirmed by our results. For the plasmid carrying the d(C)₁₈-containing insert with the d(C)₁₀ oligonucleotide, there was protection at pH 4.2-4.3 (Fig. 4a, lanes 3

* Permanent address: Institute of Molecular Genetics, USSR Academy of Sciences, Moscow 123182, USSR

† To whom correspondence should be addressed.

FIG. 2 Detection of pyrimidine dimers in the DNA strand carrying the poly-pyrimidine d(C)₁₈-containing insert. Lanes 1 and 6, control experiments without oligonucleotides (—); lanes 2 and 7, experiments with d(C)₁₀ (C); lanes 3 and 8, with d(CT)₅ (CT); lanes 4 and 9, with d(G)₁₀ (G); lanes 5 and 10, with d(AG)₅ (AG). Irradiation was performed in a buffer with 0.2 sodium acetate, pH 4.3, or with STE buffer, pH 8.0, 0.2 M NaCl, 0.1 M Tris (pH 8.0), 1 mM EDTA (pH 8.0), as indicated.

METHODS. The procedure was the same as described in Fig. 1 legend with the following changes. After the second digestion with restriction endonucleases, the small fragment was purified in a 6% polyacrylamide gel; then 0.02 µg unlabelled d(C)₁₀ and 0.05 pmol labelled fragment were mixed, and 20 µl appropriate buffer (see above) was added. After incubation for 30 min, samples were irradiated by two germicidal lamps (15 W, 254 nm) for 80 s with an intensity near 60 J s⁻¹ m⁻². Irradiated fragments were treated with 1 M piperidine (Fisher, 99.9% purity at 90 °C for 30 min) and resolved in a denaturing gel. The sequence is 5'-AGCTTGCATGCCTGCA(C)₁₈TGCAGGTGCACTCTAGAGGATCCCCGGTACCGAGCTCGAATT-3'.



and 4), whereas between pH 4.4 and 4.6 this protection disappeared. The same result was obtained for DNA carrying the d(CT)₁₆-containing insert (Fig. 4b): protection disappeared between pH 5.3 to 5.7 (lanes 7–9). The photofootprinting assay therefore allows the gradual destabilization of the intermolecular triplex within a narrow range of pH to be followed.

Why does triplex formation have such a dramatic effect on the UV-induced dimerization of duplex DNA? DNA photodamage is structure-dependent^{2,9}. Becker and Wang³ reported a 5–10-fold decrease in photodamage of the duplex after its transition from the B form to the A form. In triplexes, the duplex is in the A form¹⁰. It is evident that the protection reported here is not only due to the duplex adopting the A form. The occupa-

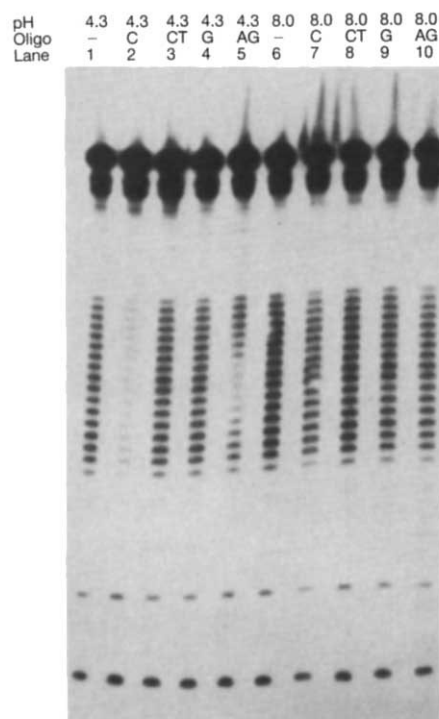


FIG. 3 Detection of pyrimidine dimers in the DNA strand carrying the poly-pyrimidine d(TC)₁₆-containing inserts. Lanes 1, 2 and 7, control experiments without an oligonucleotide (—); lanes 3 and 8, experiments with d(C)₁₀ (C); lanes 4 and 9, with d(CT)₅ (CT); lanes 5 and 10, with d(G)₁₀ (G); lanes 6 and 11, with d(AG)₅ (AG); lanes C and CT, standard Maxam-Gilbert ladders. Irradiation was performed in a buffer with 0.2 M sodium acetate, pH 4.5, or in STE buffer pH 8, as indicated.

METHODS. Plasmid pCT_{33.4} was constructed by I. K. Chung from our pTC33 plasmid carrying (CT)₁₆ inserted between *Eco*RI and *Bam*HI sites of pUC19 plasmid⁸. To obtain pCT33.4 plasmid, the small *Bam*HI-*Eco*RI fragment of pTC33 plasmid was excised, the sticky ends filled, and the fragment ligated into the *Sma*I site of pUC19 plasmid. As a result, pCT33.4 plasmid carrying two copies of the (CT)₁₆ track was obtained. The experimental procedure was the same as described in the Fig. 3 legend. The labelled strand sequence is 5'-AGCTTGCATGCCTGCAGGTGCACTCTAGAGGATCCCGATCC(TC)₁₆GAATTGATCC(TC)₁₆GAATTGGGTACCGAGCTCGAATT-3'.

tion of the major groove of DNA by an oligonucleotide, and the formation of Hoogsteen pairs with the purine strand, further hinder the internal motions in DNA so as to prevent adjacent pyrimidines from acquiring positions that favour their dimerization under UV irradiation.

We have demonstrated here protection against the formation of [6–4]-pyrimidine dimers only. But we predict that there is similar protection against the formation of cyclobutane pyrimidine dimers, because their formation also requires duplex motility².

UV irradiation is an important tool for studying DNA structure and interactions *in vivo*⁶. Our data indicate that the photofootprinting assay could be a very powerful tool for studying

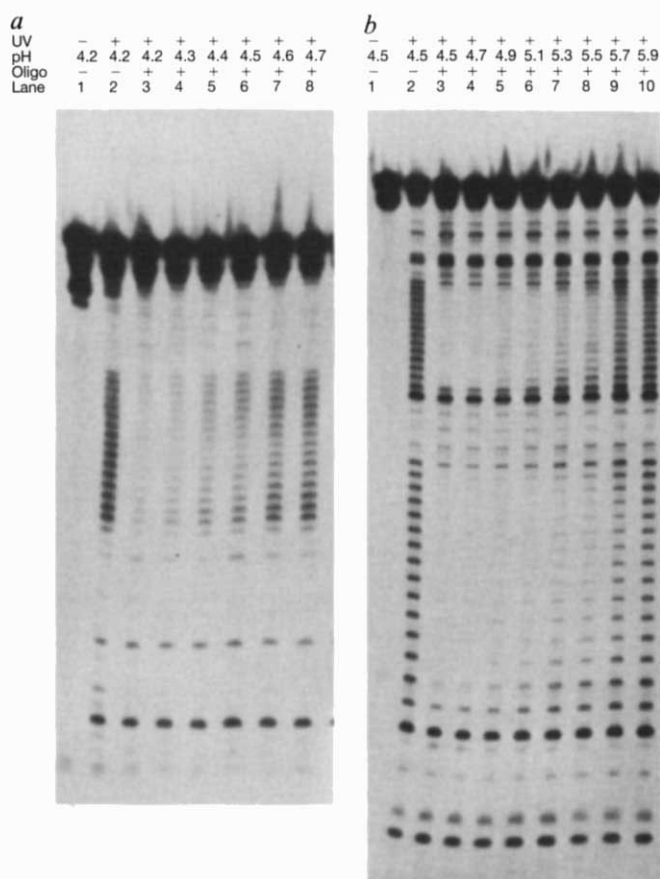


FIG. 4 Dependence on pH of pyrimidine-dimer formation in DNA strand carrying a polypyrimidine insert. **a**, The d(C)₁₈ insert. Lanes 1 and 2, control experiments without oligonucleotide at pH 4.2 (-); lanes 3-8, experiments with d(C)₁₀ at pH 4.2 (lane 3), pH 4.3 (lane 4), pH 4.4 (lane 5), pH 4.5 (lane 6), pH 4.6 (lane 7) and pH 4.7 (lane 8) (+). **b**, The d(CT)₁₆ insert. Lanes 1 and 2, control experiments without oligonucleotide at pH 4.5 (-); lanes 3-10, experiments with d(CT)₅ at pH 4.5 (lane 3), pH 4.7 (lane 4), pH 4.9 (lane 5), pH 5.1 (lane 6), pH 5.3 (lane 7), pH 5.5 (lane 8), pH 5.7 (lane 9) and pH 5.9 (lane 10) (+). The procedures were as described in legends to Figs 1 and 2.

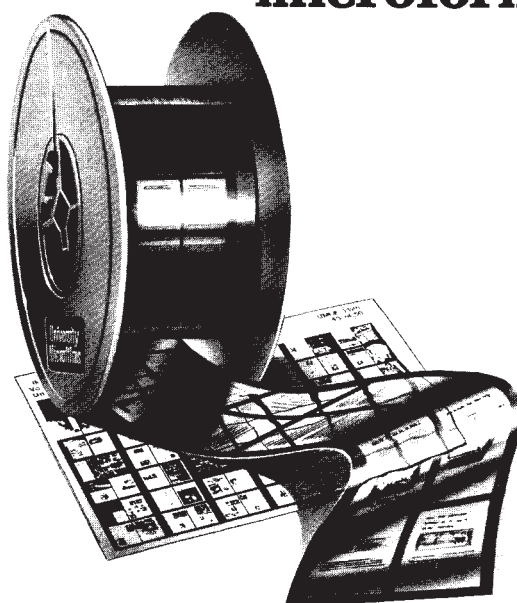
DNA triplexes. The assay could be used to detect intramolecular triplexes (H-form DNA^{7,8,11-13}) (V.I.L., M.D.F.-K. and V.N.S., manuscript in preparation). In contrast to most other methods of triplex detection (gel-electrophoresis¹⁴, chemical¹³ and enzymatic¹⁵ probing), the photofootprinting assay could be used to study unusual DNA conformations in living cells. □

Received 19 October 1989; accepted 6 February 1990.

- Gordon, L.K. & Haseltine, W. A. *Radiat. Res.* **89**, 99 (1982).
- Becker, M. M. & Wang, Z. *J. molec. Biol.* **210**, 429-438 (1989).
- Becker, M. M. & Wang, Z. *J. biol. Chem.* **264**, 4163-4167 (1989).
- Lippke, J. A., Gordon, L. K., Brash, D. E. & Haseltine, W. A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3388-3392 (1981).
- Lyamichev, V. I., Mirkin, S. M., Frank-Kamenetskii, M. D. & Cantor, C. R. *Nucleic Acids Res.* **16**, 2165-2178 (1988).
- Becker, M. M. & Wang, J. C. *Nature* **309**, 682-687 (1984).
- Lyamichev, V. I., Mirkin, S. M. & Frank-Kamenetskii, M. D. *J. biomolec. Struct. Dyn.* **5**, 275-282 (1987).
- Lyamichev, V. I. et al. *Nucleic Acids Res.* **17**, 9417-9423 (1989).
- Friedberg, E. C. *DNA Repair* (Freeman, New York, 1985).
- Arnott, S., Bond, P. J., Selsing, E. & Smith, P. J. C., *Nucleic Acids Res.* **3**, 2459-2470 (1976).
- Lyamichev, V. I., Mirkin, S. M. & Frank-Kamenetskii, M. D., *J. biomolec. Struct. Dyn.* **3**, 667-669 (1986).
- Mirkin, S. M. et al. *Nature* **330**, 495-497 (1987).
- Voloshin, O. N. et al. *Nature* **333**, 475-476 (1988).
- Lyamichev, V. I., Mirkin, S. M. & Frank-Kamenetskii, M. D. *J. biomolec. Struct. Dyn.* **3**, 327-338 (1985).
- Bernues, J., Beltran, R., Casasnovas, J. M. & Azorin, F. *EMBO J.* **8**, 2087-2094 (1989).

ACKNOWLEDGEMENTS. We thank Drs Thomas Byers, Donald Dean, Sergei Mirkin, Mark Muller and Philip Perlman, Ms Jane Tolley and In Kwon Chung.

**nature
is available in
microform.**



University Microfilms

International reproduces this publication in microform: microfiche and 16mm or 35mm film. For information about this publication or any of the more than 13,000 titles we offer, complete and mail the coupon to: University Microfilms International, 300 N. Zeeb Road, Ann Arbor, MI 48106. Call us toll-free for an immediate response: 800-521-3044. Or call collect in Michigan, Alaska and Hawaii: 313-761-4700.

☐ Please send information about these titles:

Name _____

Company/Institution _____

Address _____

City _____

State _____ Zip _____

Phone () _____

**University
Microfilms
International**

A look inside the laboratory

With a host of products that are new on the market, this week's selection includes a '386' laptop computer, a multifunction calculator, a DNA phosphatase that can be heat-killed, and a 'dry' benchtop sterilizer.

ADVANCED Magnetix has two iodinated [125 I] solution-phase **RIA kits** for the measurement of human and rat α -atrial natriuretic peptide (ANP) (*Reader Service No. 100*). Both kits offer a choice of either magnetic separation or centrifugation to separate the 'free' and 'bound' phases.



AMI's 125 I RIA kits measure α -atrial natriuretic peptide.

The human and rat α -ANP kits have a sensitivity of 2.2 and 2.7 pg α -ANP per 0.1 ml of sample, respectively. AMI supply the kits with enough lyophilized reagents for 100 assay tubes over a suggested range of 3.0 to 200 pg per 0.1-ml sample. The kits cost \$295 (US) each.

Using the **PolyATtract mRNA isolation system** and total RNA as a starting material, Promega says the mRNA fraction can be isolated free of ribosomal contamination in less than 45 minutes (*Reader Service No. 101*). The system uses a biotinylated oligo(dT)-primer to hybridize at high efficiency in solution to the 3' poly(A) region of most eukaryotic mRNA species. Using Promega's MagneSphere technology, the hybrids are captured and washed using streptavidin-bound paramagnetic particles and a magnetic separation stand. The procedure is designed to provide an enriched mRNA fraction after only a single purification cycle.

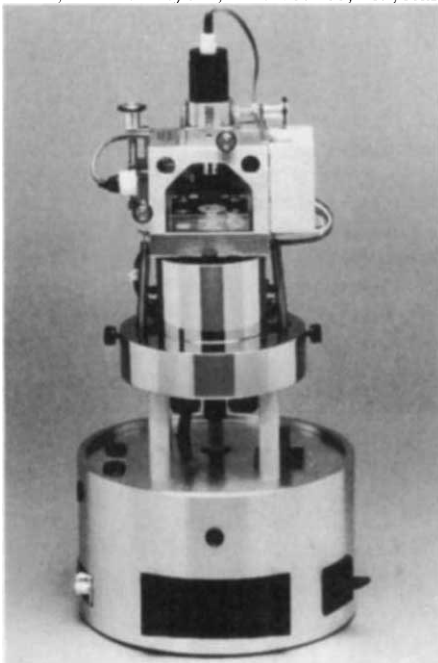
A new line of **transforming growth factors** (TGFs) produced by recombinant DNA techniques are now available from the Research Products Division of Immunodiagnostic Systems (*Reader Service No. 102*). The growth factors, which can be used to improve our understanding of the mechanisms controlling cellular proliferation, include: human platelet TGF- β 1, human tumour cell TGF- β 2 and bovine spleen TGF- β 1. Each TGF is supplied in a 1- μ g quantity. IDS points out that TGFs produced by recombinant means offer high purity, reproducible



Recombinant transforming growth factors from IDS are biohazard-free.

results and freedom from the biohazards that can be associated with TGFs derived from human and animal sources.

The NanoScope **atomic force microscope** (AFM) is being offered as an optional accessory to the NanoScope II scanning probe microscope system (*Reader Service No. 103*). The NanoScope AFM can be retrofitted to the NanoScope II and is designed for sub-nanolitre three-dimensional resolution. It can be used directly on uncoated insulating as well as conducting surfaces, in either air or liquid. Applications include the study of polymers, oxide layers, dielectrics, crystal



Digital Instrument's NanoScope atomic force microscope provides sub-nanolitre 3-D resolution.

structure and biological material, including whole cells. The basic NanoScope AFM includes a base with special electronics, an optional feedback assembly, a $0.5 \times 0.5 \mu$ scan head, and control software. When configured with optional scanners, the AFM can produce scans of areas up to $75 \times 75 \mu$. To simplify procedures, the AFM uses the NanoScope II's existing interface, image generation and analysis tools.

Portable PCs/calculators

The Lyte-Byte 5400 from Micro Express is a **laptop personal computer** (PC) that is based on the 80386SX microprocessor (*Reader Service No. 104*). Costing \$2,999 (US), the laptop PC has 1 Mbyte of RAM, a 40-Mbyte hard disk and a 1.44-Mbyte 3.5-inch floppy disk drive. In addition, it



Lyte-Byte 5400 — the portable PC from Micro Express with 386 power.

has a high-resolution (640×480 pixel) VGA gas-plasma display and up to 16 shades of gray scale on the screen. By adding a plug-in card, RAM can be extended up to 4 Mbytes. The laptop comes complete with two serial ports and one parallel port, as well as ports for an external keyboard and monitor, and 5.25-inch floppy disk drive. Lyte-Byte can be used internationally as it accepts 90-270 V a.c. Weighing just 15 lbs and measuring $14.8 \times 13.6 \times 3.8$ inches, this compact, powerful and highly portable PC comes with a sturdy carrying case and shoulder strap.

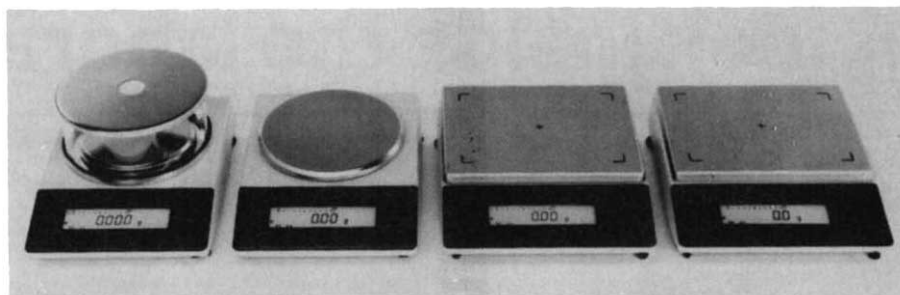
Take the headache out of record keeping and routine laboratory calculations with the latest Lab Partner **hand-held calculator** from J. & H. Berge (*Reader Service No. 105*). The \$199.95 (US) Lab Partner calculator can be used in solution preparation to calculate the amount of concentrated solution needed to prepare dilutions and the amount of material needed to fortify solutions. It can be used to make conversions between similar or

dissimilar units and to compute percentage ion from specific ion electrode p.p.m. measurements. Lab Partner can also perform centrifuge calculations, and can determine enzyme-specific activities and calculate equivalent weights. The more advanced \$279.95 (US) Lab Partner Plus has all of the above features, but also includes a mixture program for computing volumes, concentrations or specific activities with two active components or one active and one inactive component. Other features include a radioactive decay program and ninety additional memories. Both models can be linked up to an optional \$249.95 (US) 40-column Lab Printer for permanent record keeping.

The HP 48SX **scientific expandable calculator** makes the most of a calculator's dedicated user interface, while taking advantage of the storage and display capabilities of a PC (*Reader Service No. 106*). The HP 48SX features automatic unit management, which avoids tedious unit conversions and any inaccuracies that may arise as a result. The optional serial interface kit for connection to PCs provides the user with the option of using a larger monitor and QWERTY keyboard for programming the HP 48SX. The interface also provides access to the computer's printer for the generation of high-quality printouts. Also, data and programs can be stored. The enhanced graphics function is integrated with calculus functions to automatically find roots, intersections, derivatives, slopes and areas under curves. Eight different graph formats are available. The HP equation writer application allows the user to enter equations in the same form in which they appear in the textbook. The calculator has two expansion slots for RAM and ROM cards.

Balance of power

The AT Series of **analytical balances** from Mettler are designed with weighing efficiency and convenience in mind (*Reader Service No. 107*). A 2- μ g readability is provided up to 22 g with the AT20, 0.01 mg up to 205 g with the AT201, and 0.1 mg up to 405 g with the AT400. The built-in intelligence system provides fully automatic calibration technology, with sensors that register changes in the surroundings, such as temperature changes or drafts. The balances feature a large, easily accessible draft shield. At the touch of a button, the shield closes, the balance is tared, the sample weighed and the shield reopened. The DeltaTrac clock-like circular display guides the user through the weighing process, showing how much of the weighing range has been used. Other design features include switchable weights, three settings to dampen the effects of vibrations, and a RS232 interface to connect the balance to computers, printers, and LIMS.



LC Series: the next generation of laboratory balances from Sartorius.

Sartorius has launched a new line of **electronic balances** called the LC Series (*Reader Service No. 108*). The balances incorporate MC 1 technology, comprising a single-chip microcomputer designed specifically for weighing applications. The IQ mode allows the user to select the desired level of accuracy, which cuts down on weighing time. Four filters suppress vibrations and drafts, so that the balance will stabilise in about 1.5 seconds, says Sartorius. Standard application programs include differential weighing, weighing in per cent, sieve analysis, formulations, animal weighing, calculation of weights, statistics, totalling, classification/weighting-in series, counting and mass-unit conversions. To make the display easy to read at all times, the reflective backlit LCD adapts automatically to lighting conditions. Data can be relayed to peripheral devices. The LC Series of balances include a built-in data interface, automatic zero tracking, a hanger for below-balance weighing, an a.c. adapter, dust cover and draft shield on all 1.0-mg models.

Benchtop gadgetry

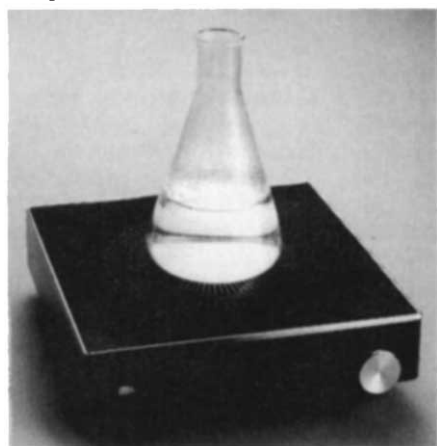
Innova is the new line of space-saving **biological shakers** from New Brunswick Scientific, which feature advanced microcomputer technology (*Reader Service No. 109*). The complete range of shakers includes a small, medium and large platform shaker, water-bath shaker, benchtop incubator shaker and a floor model controlled-environment incubator shaker. Featuring low-profile designs and a triple-eccentric drive, these models offer an extended shaking range from 25-500 r.p.m., providing, says NBS, improved oxygen transfer in the growth of microbial



Space-saving shakers from NBS.

cultures. Running times are programmable, with run parameters shown on the digital display or as chart-recorder output. Self-diagnostic indicator lights and an audible alarm alert the user to deviations from the run program.

The **infrared glass-ceramic hotplate** from Schott Gruppe provides a flameless way to heat solutions (*Reader Service No. 110*). The hotplate is available in two sizes: the 10 x 10-inch and 12 x 12-inch design. The infrared heating element can heat up to a temperature of 600 °C. One litre of water



Heat solutions the flameless way with Schott Gruppe's infrared hotplate.

will boil in less than 11 minutes, says Schott. When in use, the heating element glows orange, when not in use, it is invisible. To prevent overheating, the hotplate features automatic temperature control. For safety purposes, the indicator light will remain illuminated while the hotplate is cooling — even if the hotplate is turned off, but connected to the power source. The hotplate features an easy-to-clean enamelled-steel housing, a chemically-resistant surface and a metal ridge to contain spills.

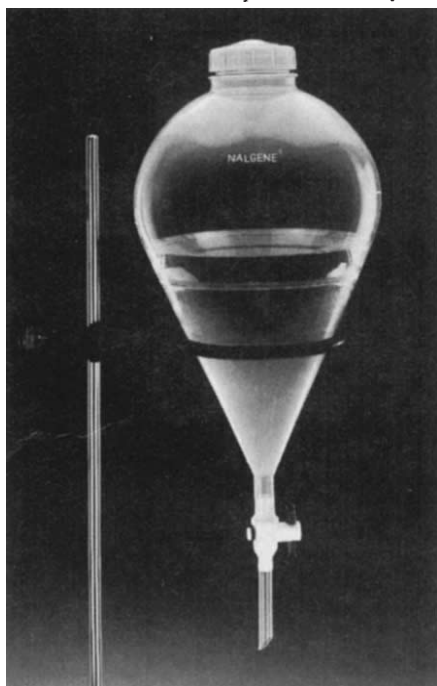
Marathon, Spark Holland's autosampler, is now configured for **automated micro LC injections**, providing, says the company, small-sample analysis and the economical use of solvents (*Reader Service No. 111*). The instrument can be equipped with either a Rheodyne micro-injector for sub-microlitre injections, or a Valco micro-injector for nanolitre quantities (60-500 nl). The minimum sample volume for the first injection is 10 μ l: repeat injections



Marathon: Spark Holland's HPLC micro-injector for small-volume analysis.

require 4 μ l. According to Spark Holland, Marathon provides a reproducibility of 0.2-0.5 per cent for 60-nl injections. It can accommodate 96 samples and comes equipped with a column oven, as standard.

For environmental analyses, Nalge has introduced a two-litre break-resistant Teflon **separatory funnel** (Reader Service No. 112). The funnel has a non-stick, non-wetting surface, which is impervious to the chemicals used in separation analyses,



Separatory funnel — ideal for radiological and hazardous separations.

says Nalge. Phase interfaces are clearly visible through the near-transparent surface. The stopcock assembly has a 4-mm bore for faster flow-through, and is designed to be leakproof up to 10 psig (vapour pressure of ether). Each \$135 (US) funnel can be autoclaved, except for the stopcock, which can be chemically disinfected.

Molecular miscellany

Epicentre Technologies has a **DNA phosphatase that can be heat killed** — HK

phosphatase (Reader Service No. 113). Isolated and purified from a bacterium that lives in Arctic seawater, HK phosphatase can be irreversibly inactivated by a brief period of heat treatment, allowing sequential steps leading to cloning or kinase labelling to be performed in a single test tube. One unit of HK phosphatase dephosphorylates 1 μ g of *Hind* III-digested pUC19 DNA in one hour, says Epicentre, when using its recommended assay protocol. The enzyme activity of HK phosphatase remains for at least three hours. After heat treatment at 65 °C for 30 minutes, less than 0.01 per cent of original enzyme activity remains. HK phosphatase can be stored at -20 °C, and is supplied in 15, 35 and 100 units for \$55, \$100 and \$240 (US), respectively. The enzyme is free of contaminating activity from proteinase, RNase, exo- and endonucleolytic DNase.

Eliminate the need for slab gels and time-consuming staining protocols with the SepraGene kits from Applied Biosystems designed for the **separation of double-stranded DNA** by capillary electrophoresis (Reader Service No. 114). Two kits are available for separating dsDNA fragments within a specific molecular weight range: SepraGene 500 for the 100 to 1,000 base-pair range (500 optimal), and SepraGene 5000 for the 1,000 to 7,000 base-pair range (5,000 optimal). The kit provides an accurate method for determining the size of unknown fragments and concentrations, says the company. The



SepraGene kits from Applied Biosystems for dsDNA analysis eliminate the need for slab gels and staining protocols.

analysis uses less than 10 nl of sample and can take less than 30 minutes from start to finish. SepraGene kits are designed to provide more than a two-fold increase in sample concentration sensitivity over ethidium bromide-stained gels, with a mass sensitivity at the femtogram level. Each kit is supplied with ready-to-use reagents, premeasured molecular weight/calibration standards, pre-assembled 50- μ m capillaries — sufficient for up to 500 analyses, says Applied Biosystems.

British Bio-technology has launched an **amplification reaction control kit** called TaqCheck (Reader Service No. 115). The use of TaqCheck is twofold: first, it pro-

vides a means of testing *Taq* polymerase and buffer constituents, and second, it provides a guide to the sensitivity of the method used. Known primer/target combinations provide a standard positive control for use in amplification reactions. Two TaqCheck kits are available, each costing £110 (UK), for the amplification of 259 and 540 base-pair fragments. Each kit consists of four tubes containing amplification primers specific for the fragment size stated, as well as sufficient plasmid DNA to provide 300, 3,000, 3×10^4 or 3×10^5 copies per 100- μ l reaction. Taq Check kits contain enough material for five standard amplification reactions at each target copy number.

New on the market from Boehringer Mannheim is a reagent called *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethammonium chloride (DOTMA) for the **transfection of DNA into mammalian cells** (Reader Service No. 116). The cationic lipid, DOTMA, forms unilamellar vesicles (liposomes) in aqueous solution following sonication. These interact spontaneously with DNA to form stable complexes that then adhere to the cell surface, fuse with the cell membrane and release DNA into the cytoplasm. Stable complexes are also formed when DOTMA and DNA are separately pre-diluted with tissue culture medium containing serum, before mixing. After the culture medium is removed, the mixture is added to cells for transfection. According to Boehringer, this procedure is gentle, avoids cytotoxic effects and over

ADVERTISEMENTS

EARTHQUAKE

Loma Prieta San Francisco Earthquake, 17th October 1989 . . . a collection of video images.

An authoritative 53 min. documentary of this major seismic event produced by the U.S. Geological Survey.

UK£15.95 or US\$25.00.

VHS format PAL or NTSC versions (PAL sent unless NTSC requested).

MASTERCARD or send cheque to: **MJP — GEOPACKS, PO Box 23, St. Just, Penzance, Cornwall, UK. TR19 7JS.** Tel. 0736 787808. Fax 0736 787880.

Papers

Bibliographic Database

For an IBM PC/AT compatible computer
£50.00 (inclusive)

Write for your free trial disk to:
**DBF Systems (N), PO Box 1374,
London W5 3XX**
Please state disk size: 3.5" or 5.25"

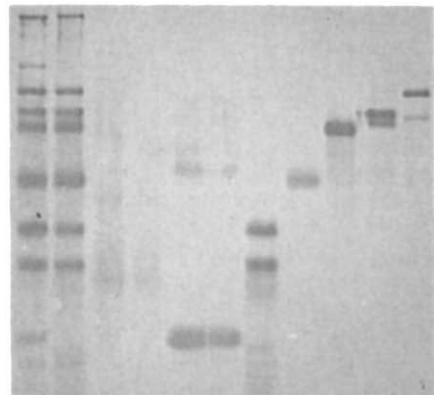
Reader Service No.47

Reader Service No.46

95 per cent of cells are transfected with DNA.

Blots and gels

Researchers can now choose from one of four new **horseradish peroxidase substrate kits** from Vector Laboratories for the detection of Western and Southern transfer blots (*Reader Service No. 117*). They are designed for use with Vectastain ABC or Elite ABC kits. The four chromagens include diaminobenzidine (DAB), chloronaphthol (4CN), tetramethylbenzidine (TMB) and aminoethylcarbazole (AEC). These substrates are convenient to use as



Western blot detection using Vector's diaminobenzidine substrate kit.

ADVERTISEMENTS

Custom DNA

Purified and Shipped in 48 hours,
Worldwide.

100 ug, \$10.00 a base for the first 15 bases,
\$7.50 for each additional base.
Delivered.

Research Genetics

1-800-533-4363

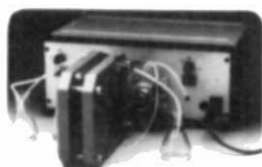
In the United Kingdom 0-800-89-1393
In Canada 1-800-533-4363

FAX 205-536-9016

Reader Service No.26

LIPOSOMES

REPRODUCIBLE - HOMOGENEOUS -
EXTREMELY RAPID



Our new LIPOSOMAT guarantees rapid (5ml within 60 minutes) preparation of uniformly sized unilamellar liposomes. Liposome size is selectable between 25 and ca. 600 nm diameter. Sample volume is normally 5-10ml or up to 200ml if used in combination with an additional instrument. Lipid concentration can be up to 300 mg/ml.

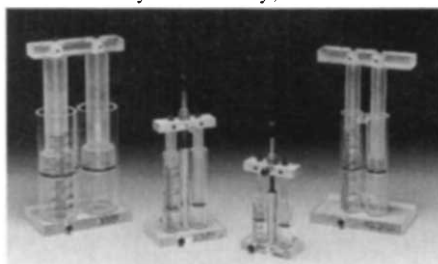
DIABORM

POB 126, D-8000 Munich 65, FRG

Reader Service No.45

all materials are supplied as concentrated stock solutions in dropper bottles. Each kit contains enough stock reagents to develop more than fifty 100-cm² blots, says Vector. The DAB substrate kit also contains nickel chloride to enhance the sensitivity, while the TMB substrate kit contains a stabiliser that makes it suitable for use with nylon and nitrocellulose blots. When used according to recommended protocols, kits can be stored for at least one year and can be used to produce permanent bands in 5 to 30 minutes. All four kits cost \$45 (US) each.

July, Inc. is offering small-volume **dual-piston gradient formers**, which have applications in electrophoresis, centrifugation and chromatography (*Reader Service No. 118*). The dual-piston patented design dispenses the fluid in each chamber at an equal rate, producing, says July, linear gradients, independent of fluid viscosity and density, in less than two

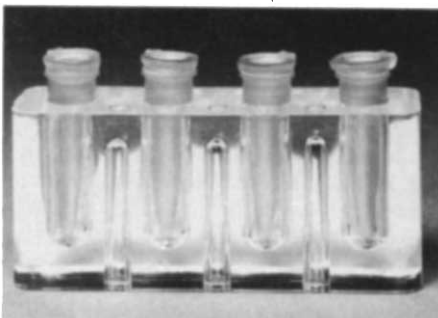


Small-volume dual-piston gradient formers from July.

minutes. Gradients can be formed automatically by connecting the gradient former to a peristaltic pump, or manually, by depressing the piston assembly. Made of acrylic plastic, gradient formers are available in 5-, 17-, 50- and 200-ml sizes for \$360, \$360, \$360 and \$385 (US), respectively. The 5- and 17-ml models have a leadscrew for dispensing small volumes in manual mode. Each gradient former is supplied with a manual, which sets out the theory, formulas and examples of exponential gradient forming.

Lab safety

Beef up safety procedures around the laboratory by reducing personal exposure to beta-emitting isotopes such as ³²P, with the Beta Blok **tube holder** from Research Products International (*Reader Service*



BPI's Beta Blok shields against beta-emitting isotopes.

No. 119). Made of solid but transparent acrylic, Beta Blok holds four 1.5-ml microcentrifuge tubes at one end and three 0.4-ml tubes at the other end.

Dispense with the need for flame, gas or chemical sterilization — Inotech has brought out a benchtop **dry sterilizer** (*Reader Service No. 120*). Available in two sizes, the Steri-250 and Steri-350, the device is designed for sterilizing instruments for tissue culture and animal surgery in 5-10 seconds. Thermoresistant glass beads in the central well of the sterilizer quickly reach a temperature of 250 °C, which, Inotech says, is suitable for



Inotech's 'dry' sterilizer eliminates the need for flames gases or disinfectants.

sterilizing all pathogens and microbial contaminants on contact. As a safety feature, the outside of the sterilizer remains cool when in use. Instruments cool to a working temperature in 30-60 seconds.

Jencons Scientific offers Klorsept **chlorine-based disinfectant** in tablet and granule form (*Reader Service No. 121*). The active ingredient, sodium dichloroisocyanurate, is effective against bacteria, fungi, spores and viruses, including AIDS and hepatitis B, says Jencons. The effervescent tablets provide an accurate measured dose and are in a convenient form to make up solutions. Klorsept granules can be used to mop up spillages around the laboratory: each 500-g container can absorb up to 10 litres of spillage. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.